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ABSTRACT

Three experiments were conducted to investigate the relationships of plasma cortisol, CBG concentration, and CBG mRNA expression during development and heat and social stress in pigs. The first experiment was conducted to develop and characterize a porcine CBG cDNA probe in order to examine porcine CBG mRNA expression in some major tissues from the postnatal pig. The reverse transcriptase-polymerase chain reaction (RT-PCR) was conducted to develop the porcine CBG cDNA probe from liver total RNA extracted from pigs on 40 days of age. The RT-PCR product was subcloned into the pGEM vector and subjected to the treatment of restriction enzymes and DNA sequencing. Northern blot analysis was conducted using total RNA extracted from samples (~ 200 mg) of liver, lung, kidney and whole adrenal tissue that were collected from neonatal pigs either 3 (n = 2) or 40 (n = 2) days of age. A 500 base pair (bp) partial porcine CBG cDNA encoded 166 amino acids and had 83%, 78%, and 77% homology to a 494 bp nucleotide sequence of sheep, human, and rabbit, respectively. The deduced amino acids sequence of the partial porcine CBG showed 77%, 62%, 60% and 51% homology to sheep, human, and rabbit, and rat CBG sequences, respectively. An approximately 1.53-kilobase CBG mRNA was detected only in the liver tissue

The second experiment was conducted to evaluate the relationships among hepatic CBG mRNA expression and plasma concentrations of cortisol and CBG during prenatal and postnatal periods in the pig. Blood and liver tissue

were collected from fetal pigs (n = 7-14 per age) on day 50, 70, 80, 90, and 104 of gestation, as estimated by fetal crown-rump length, and from postnatal pigs (n = 8 per age) on day 1, 3, 10, 20, 30, and 40 following birth. Plasma cortisol and CBG concentrations were determined by a radioimmunoassay (RIA) and an enzyme-linked immunosorbent assay (ELISA), respectively. CBG mRNA expression was determined from liver total RNA by Northern blot analysis and expressed relative to β-actin mRNA expression level. In fetal pigs, CBG mRNA expression was highest ($P < 0.01$) on day 50 compared to day 90 exhibiting a negative relationship ($r = - 0.63$, $P < 0.01$) with estimated gestation age. Plasma CBG concentrations were correlated ($r = 0.34$, $P < 0.05$) to CBG mRNA levels. Plasma cortisol concentrations were not different over this same period. In postnatal pigs, CBG mRNA expression increased ($P < 0.01$) from day 3 to day 40. Plasma CBG concentration increased ($P < 0.01$) from day 1 (6.1 ± 3.4 μg/ml) to day 10 (15.1 ± 3.7 μg/ml). Plasma cortisol concentrations remained constant.

The third experiment was conducted to examine the relationships among plasma cortisol and CBG levels, and hepatic CBG mRNA expression in pigs subjected to elevated environmental temperature in conjunction with establishing social hierarchy. Twenty-four pigs (three or six pigs per litter) were weaned at 25 days of age and housed by litter for 2 weeks at 23 ± 2°C. On day 0, animals were weighed and placed under general anesthesia for collection of blood (10 ml) and liver tissue (~100 mg). On day 1, three pigs of similar weight (23 ± 0.9 kg) but from different litters were allotted to eight nursery pens within two environmentally controlled rooms (12 animals/room). From days 1 to 7

(treatment period), one room was maintained at 23 ± 2°C (control, CON) and the other at 33 ± 2°C (heat treatment, HEAT). From days 8 to 14 (recovery period), both rooms were maintained at 23 ± 2°C. Animals were videotaped for 72 hours beginning on days 1 and 8 to document behavioral changes in response to room temperature and to determine social order. Blood and liver tissue were collected again on days 7 and 14. Plasma haptoglobin increased ($P < 0.05$) from 467 ± 123 μg/ml on day 0 to 763 ± 113 μg/ml on day 7 in HEAT pigs. Plasma cortisol and CBG decreased ($P < 0.05$) from 99.3 ± 8.3 ng/ml and 11.4 ± 1.1 ug/ml on day 0 to 85.1 ± 8.3 ng/ml and 9.9 ± 1.1 μg/ml on day 7 in HEAT pigs, respectively. Hepatic CBG mRNA level and neutrophil:lymphocyte ratio were not affected ($P > 0.1$) by treatment. HEAT pigs displayed increased ($P < 0.01$) drinking but reduced feeding ($P < 0.01$) and lying in contact with other pigs ($P < 0.05$) behaviors. Average daily gain of body weight (ADG) tended ($P = 0.06$) to be lower for HEAT (0.64 ± 0.06 kg/d) compared to CON (0.82 ± 0.06 kg/d) pigs. During the recovery period, HEAT pigs had similar ($P > 0.1$) ADG, plasma cortisol, CBG, haptoglobin, and drinking and feeding behavior but increased ($P < 0.01$) lying with contact behaviors compared to CON pigs. Measured physiological and behavioral responses were not related to social status

In summary, a 500 bp partial porcine CBG cDNA developed from pig liver total RNA had high homology with CBG cDNA from human, sheep, and rabbit. Liver was the primary source of CBG biosynthesis in the postnatal pig. In fetal pigs, plasma CBG level was mainly determined by hepatic CBG mRNA expression that was inversely related to gestational age. In postnatal pigs,

hepatic CBG mRNA was directly related to age but plasma CBG levels did not appear to be determined by hepatic CBG mRNA expression alone. Also, reduced circulating levels of cortisol and CBG in pigs following a 7-day exposure to elevated temperature was not be attributed to changes in hepatic CBG mRNA expression.

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INTRODUCTION

The swine industry experiences a high rate of neonatal mortality. Neonatal mortality is associated with maternal care, low birth weight, nutritional deprivation, and a number of environmental stressors (Varley, 1995). It has been known that cortisol plays an important role in preparing the fetus for birth (Liggins, 1994), induction of parturition (First and Mose, 1979), postnatal development of various organ systems (Henning, 1981), and adaptation to stress (Munck et al., 1984). Therefore, changes in plasma cortisol levels may be closely related to neonatal mortality and well-being in pigs.

Cortisol exists in both unbound and protein-bond forms in circulation. Corticosteroid-binding globulin (CBG) is the major transport glycoprotein that binds cortisol with high affinity in circulation (Ballard, 1979). CBG alters the rate of cortisol disappearance (Migeon et al., 1968) and influences on pool size and distribution of cortisol in circulation (Bright, 1995). Therefore, CBG in circulation can regulate the plasma concentration as well as bioavailability of cortisol (Bright, 1995).

In pigs, plasma CBG levels fluctuate during prenatal and postnatal development, which may influence distribution as well as bioavailability of plasma cortisol. Various stressors also affect plasma CBG levels (Tinnikove, 1993; Fleshner et al., 1995; Alexander and Irvine, 1998). Plasma CBG level is determined by its biosynthesis, metabolic degradation and/or transfer to extravascular spaces. However, it is still unclear how plasma CBG level is

regulated during development or stressful situations, and how the changes in plasma CBG level affect plasma cortisol levels. Understanding the mechanisms that regulate plasma CBG level may be useful to determine the distribution and bioavailability of plasma cortisol.

Therefore, this study was conducted to investigate the relationships of plasma cortisol and CBG levels and CBG biosynthesis during development and heat and social stress in pigs. Specifically, the objectives were:

1. To develop and characterize porcine CBG cDNA, and to examine the porcine CBG mRNA expression in some major tissues from the postnatal pig.
2. To evaluate the relationships among hepatic CBG mRNA expression and plasma concentrations of cortisol and CBG during the prenatal and postnatal periods in the pig.
3. To examine the relationships among plasma cortisol and CBG levels, and hepatic CBG mRNA expression in pigs subjected to elevated environmental temperature in conjunction with establishing social hierarchy.

CHAPTER 1. LITERATURE REVIEW

General functions of glucocorticoids

Glucocorticoids, primarily cortisol in the pig, are steroid hormones that are produced by the cells of the adrenal cortex in response to appropriate stimuli and are essential in life (Norman and Litwack, 1997). Glucocorticoids act on different cells in different ways and can regulate the expression of certain proteins by transcriptional actions (Norman and Litwack, 1997).

The effects of cortisol on glucose and protein metabolism are grossly anabolic in the liver, and catabolic and anti-anabolic in certain other tissues (Exton, 1979). Cortisol increases glucose production in the liver through induction of gluconeogenic enzymes and increased availability of substrates. Cortisol induces the breakdown of both protein and nucleic acids and decreases protein synthesis in muscle (Exton, 1979). In adipose tissue, cortisol stimulates lipolysis and promotes the lipolytic effect of epinephrine and increases the production of lactate (Baxter and Rousseau, 1979). Thus, cortisol acts to mobilize energy sources during prolonged inaccessibility to an immediate energy substrate, especially glucose (Baxter and Rousseau, 1979).

Cortisol is needed to maintain normal vascular integrity and responsiveness and the volume of body fluids (Kaplan, 1992). In the absence of cortisol, abnormal vasodilation occurs, so that even without an external loss of fluid, the filling of the vascular bed is reduced and blood pressure falls.

Cortisol blocks the reactions to various injuries and foreign substances involving multiple antiinflammatory and immune responses. The mechanisms for immunosuppressive and antiinflammatory effects of cortisol include stabilization of lysosomal membranes so that proteolytic enzymes are not released, decrease in permeability of capillaries so that less plasma and fewer cells enter inflamed areas, depression of phagocytosis by white blood cells, and suppression of thymus-derived lymphocytes (Boumpas et al., 1993). Cortisol also suppresses synthesis of several key proteins, which accentuate the inflammatory process including interleukin-1, and stimulates synthesis of others that are antiinflammatory including lipocortins that inhibit the generation of eicosanoids (Kaplan, 1992).

Cortisol modulates perception and emotion. This is usually recognized only in disease such as Cushing's syndrome and Addison's syndrome (Baxter and Rousseau, 1979). In Cushing's syndrome with cortisol excess, initial euphoria but subsequent depression is common and the threshold for seizure activity may be lowered. Addison's syndrome with cortisol deficiency involves the accentuated sense of taste, hearing, and smell.

Preceding parturition, fetal organs undergo accelerated maturation which facilitates the transition from intrauterine to extrauterine life (Liggins, 1994). Cortisol is important for the maturation of various fetal organs including the small gut, liver, lung, adrenal, and kidney. Prenatal treatment with corticosteroid in rats elicits a precocious appearance of jejunum sucrase (Celano et al., 1977). Alkaline phosphatase is also induced in fetal rabbits (Lee et al., 1976). In fetal

sheep, a relationship of prenatal rise in liver glycogen with the prenatal rise in cortisol was prevented by fetal hypophysectomy or adrenalectomy (Barnes et al., 1978). In the immature fetal lung (Liggins, 1994), cortisol stimulates surfactant synthesis and secretion, connective tissue maturation, alveolar epithelial differentiation, lung liquid resorption, glycogeneolysis and production of antioxidant enzymes. Treatment of fetal sheep with cortisol increased glomerular filtration rate and the tubular resorption of sodium (Hill et al., 1988). Cortisol also enhanced the adrenal response to adrenocorticotrophic hormone in fetal sheep (Challis et al., 1985).

Although stress stimulates a rapid increase in secretion of glucocorticoids, it remains controversial as to what purpose glucocorticoids serve at such times. Glucocorticoids inhibit the production and/or actions of a variety of intercelluar mediators such as interferon (Rytel and Kilbourne, 1966), lymphocyte activating factor (Snyder and Unanue, 1982), T cell growth factor (Gillis et al., 1979), prostaglandin (Russo-Marie et al., 1979), bradykinin (Newcombe et al., 1977), serotonin (Tsurufuji et al., 1979), histamine (Daeron et al., 1982), and β-endorphin (Simantov, 1979). These mediators are components of normal physiological defense mechanisms and can themselves cause damage and endanger survival of the organism if they are activated for too long (Munck et al., 1984). Stress can impinge on the organism and threaten homeostasis through tissue damage, metabolic and neural disturbances (Munck et al., 1984). Normal physiological defense reactions can restore homeostasis through mechanisms involving stress-induced secretion of lymphokines, hormones, neuropeptides and

other mediators (Munck et al., 1984). Therefore, Munck et al. (1984) proposed that stress-induced increases in glucocorticoid levels protect not against the source of stress itself but rather against the body's normal reactions to stress, preventing those reactions from over reacting and themselves threatening homeostasis.

Regulation of glucocorticoid production

The glucocorticoids are produced predominantly in the fasciculata of the adrenal cortex (Norman and Litwack, 1997). The production of glucocorticoids results from the activation of the hypothalamic-pituitary-adrenal (HPA) axis. The regulation of glucocorticoid secretion from the adrenal cortex depends on a linkage between the hypothalamus and the pituitary gland. Corticotrophin-releasing hormone produced in hypothalamic neurons stimulates the production of adrenocorticotrophic hormone (ACTH) from the anterior pituitary (Saffran et al., 1955). ACTH stimulates the synthesis and release of steroids from the adrenal cortex by promoting the uptake of cholesterol and its enzymatic conversion to cortisol and corticosterone (Matteri et al., 2000).

When glucocorticoids are produced in response to ACTH, it has negative effects on the hypothalamus and anterior pituitary (Norman and Litwack, 1997). Feedback inhibition of ACTH is very fast, and feedback on the hippocampus may shut off further electrical activity responsible for the release of CRH and the

subsequent release of ACTH. These actions are mediated by glucocorticoid receptors located in these cells (Norman and Litwack, 1997).

Distribution of cortisol in plasma

Cortisol exists in plasma in both unbound and protein-bound forms (Ballard, 1979). The major cortisol binding proteins in plasma are corticosteroid-binding globulin (CBG) and albumin. Free cortisol diffuses passively through target cell membranes and binds to a soluble intracelluar receptor to initiate an action (Norman and Litwack, 1997). CBG-bound cortisol constitutes a pool of readily available cortisol and albumin-bound cortisol which may supplement the free pool of cortisol within tissues because of the rapid dissociation of cortisol from albumin (Siiteri et al., 1982). Although CBG is present in plasma at low concentrations, CBG is a major binder of cortisol due to its high affinity for this particular ligand. In contrast, albumin is present at high concentration and has a much lower affinity (Siiteri et al., 1982). The normal concentration of CBG in humans is 0.71 uM with an association constant for cortisol at 3.0×10^7 L/mol. In contrast, plasma albumin binding capacity is 500 uM with an association constant for cortisol at 0.5×10^4 L/mol (Ballard, 1979). In spite of the large concentration difference between CBG and albumin in plasma, the percentage of cortisol bound to CBG (78%) is significantly higher than that bound to albumin (15%) (Ballard, 1979).

The distribution of unbound, CBG-bound, and albumin bound cortisol in several species was calculated from the CBG-binding parameters. Plasma cortisol levels were measured to evaluate the potential availability of cortisol to target tissues (Gayrard et al., 1996). Plasma cortisol concentrations varied from 0.02 to 0.05 uM (ewe, dog, cow), 0.1 to 0.6 uM (horse, human, cynomolgus monkey), and 1.6 uM (squirrel monkey). However, the distribution of unbound (6 to 14%), CBG-bound (67 to 87%), and albumin-bound (7 to 19%) fractions of cortisol were similar between species.

This pattern of cortisol distribution may vary with age. In the newborn guinea-pig the unbound, CBG-bound and albumin-bound fractions were 20, 62, and 18%, respectively (Dalle et al., 1980). By day 20 of age the unbound fraction was 6 %, the CBG-bound fraction was 88 % and the albumin-bound fraction was 6%. Kattesh and coworkers (1990) reported that unbound, CBG-bound, and albumin-bound cortisol fractions in the pig were 30%, 40%, and 30% at birth and 15%, 54%, and 31% on day 42, respectively. Unbound cortisol appears to be high at birth and decreases with age. During early postnatal development this change in distribution of cortisol may have an important role in the maturation and adaptation of the animal (Henning, 1981).

Analysis of CBG structure and CBG gene

The CBG molecule is associated with the alpha-globulin area in the electrophoretic separation of human plasma proteins (Slaunwhite and Sandberg,

1959). CBG migrates as a 50-60 kilodalton macromolecule on polyacrylamide gel electrophoresis under denaturing conditions (Kato et al., 1988). CBG was identified as a monomeric glycoprotein with only one steroid binding site per molecule (Mickelson et al., 1982).

The CBG molecule is generally rich in methionine and contains very few cysteine residues (Hammond, 1990). It also contains an abundance of negatively charged amino acids that account in part for its acidic isoelectric point (pI, 3.4 to 4.2) (Bernutz et al., 1979). Variations in carbohydrate composition account for heterogeneity in its electrophoretic mobility and pI (Kato et al., 1988). Chemical analyses of human CBG have indicated that it is comprised five N-acetyllactosamine-oligosaccharides per molecule, of which three are biantennary and two are triantennary (Akhrem et al., 1982). The heterogeneity of the carbohydrate moieties may be more complex and reflect variations in glycosylation induced by different hormonal and physiological conditions (Hammond, 1990). The presence of carbohydrate influences the half-life of plasma CBG, and removal of sialic acid residues by neuraminidase increases the clearance of CBG by the asialo-glycoprotein receptor of the liver (Hossner and Billiar, 1981). Almost complete deglycosylation does not affect its steroid binding properties (Mickelson et al., 1982). However, variations in carbohydrate composition may modify the interaction between CBG and target cell membrane (Rosner, 1990; Avvakumov et al., 1988), and may influence any biological response (Nakhla et al., 1988). Phylogenetic comparison of CBG primary structures may reveal the location of conserved consensus sites for attachment

of carbohydrate moieties that may be physiologically important (Avvakumov et al., 1988). Affinity-labeling with 6-bromo-pregesterone has indicated that a cysteine in human CBG is located within the steroid binding site (Khan and Rosner, 1977).

A number of CBG complementary DNAs (cDNA) from human liver and lung libraries have been isolated by production of specific antisera against human CBG, revealing the primary structure and molecular composition of the protein (Hammond et al., 1987). Based on the cDNA-deduced primary structure of human CBG, it is composed of 383 amino acids, and has a polypeptide molecular weight of 42,646 (Hammond et al., 1987). There is a consensus sequence for N-glycosylation at residue 9 (asparagine) in the sequence of the mature protein (Hammond et al., 1987).

Human CBG cDNA has been used to isolate cDNAs for CBG from other species. Smith and Hammond (1989) isolated CBG cDNA from a lambda-gt liver cDNA library and determined the primary structure of rat CBG. The protein contains 374 amino acids (molecular weight = 42,196) and six consensus sites for N-glycosylation. There was a 60 % homology over 383 residues to that of human CBG. The single cysteine in rat CBG also corresponded to one of two cysteines in human CBG. Similarly, Seralini and coworkers (1990) demonstrated that the cDNA-deduced primary structure of rabbit CBG contains 383 amino acids (molecular weight = 42,326), including three cysteine residues and four sites for N-glycosylation.

Phylogenetic comparison of CBG primary structures of several species has indicated that the degree of sequence similarity is relatively poor as compared to many other proteins (Hammond et al., 1991). In this regard, there are three deletions of one or more amino acids in rat CBG sequence compared to human and rabbit proteins. These deletions are located in regions that are not well conserved, and are therefore probably not biologically very important. One of these deletions is adjacent to an intron/exon junction in the human gene (Underhill and Hammond, 1989). When the human, rat, and rabbit CBG sequences are compared, it is evident that only two of the N-glycosylation sites have been retained throughout evolution (Hammond et al., 1991). These consensus sites are located in highly conserved regions of the protein, and may therefore be functionally very important.

Kattesh and Roberts (1993) isolated and purified porcine CBG from serum using affinity chromatography and HPLC-DEAE anion exchange techniques. SDS-PAGE analysis of porcine CBG revealed that the protein consists of two polypeptides of approximately 54 and 59 kDa, and both have significant amino acid homology (>50%) to that of rabbit, human and hamster CBG for the first 38 amino acids.

Interestingly, human CBG is structurally related to several members of the serine protease inhibitor (SERPIN) superfamily including α1-proteinase inhibitor (A1-PI) and α1-antichymotrypsin (A1-ACT) (Hammond et al., 1987). This group of proteins is thought to have arisen from a common ancestral gene by a process of gene duplication and may be divided into subgroups based on protein

sequence similarity (Underhill and Hammond, 1989). Most members of the SERPIN superfamily contain a functional domain that interacts with a specific serine protease produced by activated neutrophils. It has been demonstrated that neutrophil elastase cleaves the CBG molecule in a position that is closely aligned with the proposed elastase binding site on A1-PI, resulting in a dramatic reduction in CBG steroid binding activity (Pemberton et al., 1988). Elastase cleavage of CBG appears to take place naturally on the surface of activated neutrophils from patients with acute inflammatory diseases (Hammond et al., 1990b), suggesting a physiological role for CBG in the delivery of cortisol to sites of inflammation.

Underhill and Hammond (1989) determined the structure of the human CBG gene using restriction endonuclease maps of human placental DNA and cloned genomic DNA. The human CBG appears to be encoded by a single gene that comprises five exons distributed over approximately 19 kilobases. Typical of many eukaryotic promoters, sequences that resemble TATA and CAAT-box motifs are centered 28 bp and 73 bp upstream from the origin of transcription, respectively. In addition, six highly conserved sequence elements, responsible for efficient, liver-specific expression of the mouse albumin gene, are located within the first 200 bp of the 5' flanking region. Recently, it has been demonstrated that the gene encoding human CBG is a part of a cluster of six serine protease inhibitor (serpin) genes located on human chromosome 14q32.1 (Rollini and Fournier, 1999a). The activity of the human serine protease inhibitor

gene encoding CBG is regulated by hepatocyte nuclear factors 1 alpha and 4(HNF-1alpha and HNF-4) (Rollini and Fournier, 1999b).

Biosynthesis , distribution, and metabolism of CBG

The liver has been identified as the major site of CBG biosynthesis in the guinea pig (Perrot-Applanat et al., 1981), human (Khan et al., 1984), rat (Smith and Hammond, 1989), rabbit (Seralini et al., 1990), hamster (Lin et al., 1990), fetal sheep (Jacobs et al., 1991), and mouse (Scrocchi et al., 1993). The nature of CBG-producing cells was studied in guinea-pig liver by immunocytochemistry. CBG-stained hepatocytes were more numerous in the peripheral regions of the lobules and around the portal space. The rough endoplasmic reticulum was identified as the site of CBG synthesis in the guinea-pig hepatocytes (Perrot-Applanat et al., 1981). Also, CBG has been successfully produced and secreted by hepatocytes in culture (Khan et al., 1984; Rosener et al., 1984). Khan and coworkers (1984) demonstrated that a human hepatoma cell line synthesized and secreted a protein that was functionally, as well as physically and immunologically, the same as CBG. Elfahime and coworkers (1992) examined enriched populations of hematopoietic and parenchymatous cells prepared from fetal liver for their abilities to accumulate CBG mRNA, and showed that the homogeneous hematopoietic cell fraction (virtually free of hepatocytes) failed to accumulate CBG mRNA while the parenchymatous cell fraction did express the CBG gene.

Most probably, however, CBG synthesis does not occur exclusively in the liver. The availability of species-specific cDNAs for CBG allowed for the detection of CBG mRNA by Northern blot analysis of spleen and ovary RNA from adult rabbits (Seralini et al., 1990) and kidney and pituitary RNA from fetal sheep (Berdusco et al., 1995). Although the levels of CBG mRNA in these tissues are relatively low when compared to that measured in adult liver samples (Seralini et al., 1990), the biosynthesis of CBG in these extra-hepatic tissues may influence the local bioavailability of cortisol (Scrocchi et al., 1993).

CBG is primarily localized in the blood plasma compartment. However, the presence of CBG is not confined to the intravascular volume. Indeed, significant concentrations of CBG have been found in lymph, intraperitoneal fluid, follicular fluid, amniotic fluid, milk and breast cyst fluid (Heyns and Coolens, 1987). Whereas CBG is low, but detectable in saliva (Hammond and Langley, 1986). In rat tissues, the presence of CBG in some nonhepatic cell types has been demonstrated by immunocytochemical analysis (Kuhn et al., 1986).

It has been shown that asialo-transcortin is very rapidly cleared by the liver (Hossner and Billiar, 1981). Transcortin is the name of CBG in human (Slaunwhite, Jr. and Sandberg, 1959). Therefore, the hepatic clearance of asialotranscortin through an asialo-glycoprotein receptor system may constitute an important pathway (Ashwell and Morell, 1974).

Species differences in the half-life of CBG may be related to differences in sialic acid content of the proteins (Seralini, et al., 1989). In comparing the clearance rate of CBG in infant and adult rats, the half-life of CBG in infants (~6.9

hours) was consistently less than that in adults (~ 14.5 hours), and the differences may be due to an interaction with a serine protease rather than the composition of the protein (Smith and Hammond, 1991). The half-life of rabbit CBG (13 hours) (Seralini, et al., 1989) was longer than half-lives of either human CBG (6.8 hours) or rat CBG (4-5 hours) when injected into ovariectomized hamsters and rats (Hossner and Billiar, 1981).

Roles of CBG

It has been generally accepted that free steroid diffuses passively through target cell membranes and binds to a soluble intracelluar receptor, and the steroid-receptor complex modifies the chromatin transcriptional activity in the nucleus (Siiteri et al., 1982). On the other hand, a variety of extracellular binding proteins enhance the solubility of steroid hormones in biological fluids and facilitate their transport from steroidogenic tissues to target cells (Hammond, 1990). CBG binds cortisol with remarkably high affinity and specificity compared to albumin (Ballard, 1979). As a result, CBG not only transports cortisol but also modulates its bioavailability (Siiteri et al., 1982), and may participate directly in the delivery of cortisol to certain cells by the interaction with plasma membrane receptors (Rosner, 1990).

The role that CBG serves in regulating the bioavailability of cortisol has been demonstrated in pregnant women (Rosenthal et al., 1969). Although unbound cortisol levels rose considerably during pregnancy, the percentage of

cortisol distributed among CBG, albumin and the unbound compartments remained almost constant throughout pregnancy, and was very similar to that in normal women (Rosenthal et al., 1969). Therefore, it appears that the concomitant rise of CBG during pregnancy keeps the physiologically available cortisol level low enough so as not to produce Cushing's syndrome.

CBG also serves as a relatively inert reservoir in which cortisol is protected from metabolism and excretion, but is still available by dissociation to provide sufficient hormone (Ballard, 1979). Leeper and coworker (1988) demonstrated that the increase in plasma corticosterone concentration in the rat during the third week of life resulted from a decrease in metabolic clearance rate (MCR) for corticosterone, which was affected through the increasing concentration of CBG. On the other hand, the authors also showed that the half-life of bound corticosterone was less than that of free corticosterone, suggesting that bound corticosterone may be preferentially metabolized by a mechanism for the internalization of CBG through receptor-mediated endocytosis in the target tissues. Specific binding sites for CBG were detected on membranes prepared from rat spleen (Singer et al., 1988).

During inflammation CBG responds as an acute phase negative protein (Sauv et al., 1980). In pigs, the acute phase response following inflammation is accompanied by a dramatic increase in circulating concentrations of acute phase proteins including acid soluble glycoprotein, haptoglobin, C-reactive protein, and α1-acid glycoprotein (Eckersall, et al., 1996). However, serum CBG levels drop rapidly during artificially-induced inflammation (Savu et al., 1980). CBG is

specifically cleaved by neutrophil elastase (Hammond et al., 1990), and this promotes a conformational change in the protein, which disrupts the steroid binding site and releases cortisol (Pemberton et al., 1988). During inflammation, the interaction of CBG with elastase on the surface of neutrophils results in the release of glucocorticoids directly to the activated neutrophils (Hammond et al., 1991a). These glucocorticoids down-regulate neutrophil activity by decreasing the production of chemotactic factors and other products that are involved in the inflammatory process such as prostaglandin(Hammond et al., 1991b).

Ontogeny of CBG

Plasma CBG concentration undergoes dramatic changes during development. The variation in plasma CBG levels modifies the bioavailability of cortisol, which influences maturational events during development (Henning, 1981; Liggins, 1994).

In the fetal rat, plasma CBG concentrations reach about half the level observed in the maternal circulation between 17 and 19 days of gestation, but decline to very low levels at birth (Smith and Hammond, 1991). In the neonatal rat, plasma CBG concentrations are uniformly low through the first 9 days after birth, and then begin to rise again until a plateau is reached on approximately day 24 (Henning, 1978). Smith and Hammond (1991) compared the levels of serum CBG and hepatic CBG mRNA from day 15 of gestation until 12 weeks following birth. Hepatic CBG mRNA in fetal rats was exceptionally high on day

15 of gestation, but declined to very low levels at birth (day 21). Hepatic CBG mRNA levels in neonatal rats were barely detectable 1 week after birth and reached adult CBG mRNA levels by 3 weeks of age.

Dalle and coworkers (1980) demonstrated that both age and sex-related changes in the binding capacity of CBG for cortisol occur in the guinea pig during development. CBG binding capacity for cortisol in plasma was highest at birth followed by a consistent and significant decrease by day 10. On day 20, CBG binding capacity in the male (0.74 umol/l) was not different from that measured on day 10. However, the female guinea-pig measured on day 20 had significantly higher CBG binding capacity (1.6 umol/l) than that found on day 10 (0.7 umol/l), which was significantly higher than that of males at the same age.

In the fetal rabbit, plasma CBG level was low on day 11, reached a maximum on about day 22, and then decreased to very low levels at term (Seralini et al., 1990). Hepatic CBG mRNA concentration in the same animals exhibited a profile similar to that observed for fetal serum CBG levels (Seralini et al., 1990).

In the chronically catheterized ovine fetus, plasma corticosteroid binding capacity (CBC) rose from 23.3 ng/ml at day 115 to 86.5 ng/ml at term and then decreased rapidly after birth (Berdusco et al., 1995). Using Northern blotting, the relative levels of CBG mRNA in the ovine fetal liver increased significantly at day 140 but declined at term and in newborn lambs (Berdusco et al., 1995). Therefore, at least until day 140 of gestation the rise in plasma CBC appears to be associated with an increase in hepatic CBG mRNA levels.

Kattesh and Roberts (1993) documented the changes in plasma CBG concentrations in the pig measured at various ages from 3 to 126 days of age using enzyme-linked immunosorbent assay (ELISA) procedures. The CBG concentration decreased from day 3 (227.2 μg protein /L) to day 7 (195.6 μg protein /L). The concentration of CBG was significantly higher on day 28 (276.6 μg protein /L) reaching a plateau on day 42 (351.0 μg protein /L). The increase in CBG levels around 28 days of age reflects corresponding changes in the percent distribution of cortisol among protein bound and free forms in the pig as documented earlier (Kattesh et al., 1990).

Factors affecting plasma CBG level

Gender differences in the circulating concentration of CBG have been noted. Serum CBG level in the adult female rat were 2.5-fold higher than that in the male (Jasson et al., 1989: Mataradez et al., 1992). Likewise, hepatic CBG mRNA content in the adult female was three fold higher than that of the adult male (Smith and Hammond, 1989). This sexual dimorphism in CBG gene expression may be attributed to sex differentiation of growth hormone secretion induced by the secretion of androgens and estrogen, but also by androgen imprinting of the neonatal male rats (Jansson et al., 1984; Jansson et al., 1989).

Ottenweller et al. (1979) demonstrated circadian rhythm of plasma corticosterone binding activity (CBA: an index of CBG activity) in rats and mice. The plasma CBA rhythm was bimodal in rats with peaks at 1000 and 1800 h and

unimodal in mice with peak level from 0100 to 0500 h. The plasma CBA rhythms appear to be related to the circadian rhythms of both locomotor activity and plasma corticosteroid concentration. It has been also demonstrated that a diurnal variation in CBG in the rat is the result of the diurnal variation in glucocorticoid levels (Hsu and Kuhn, 1988).

CBG concentration in the gestating female shows species-specific variation. In women (Rosenthal et al., 1969), rabbits (Seralini et al., 1990) and hamsters (Lin et al., 1990), plasma CBG is considerably elevated during pregnancy. In contrast, pregnant rat CBG levels remain almost unaltered (Smith and Hammond, 1989), and the mare shows a significant decrease in plasma CBG binding capacity throughout pregnancy which may be due to an increase in CBG uptake by tissues (Martin and Silberzahn, 1990).

Various hormones are involved in the regulation of circulating concentrations of CBG. Glucocorticoids decreased serum CBG and hepatic CBG mRNA levels in the adult rat (Smith and Hammond, 1992). In fetal sheep, glucocorticoid treatment increased plasma CBG and hepatic CBG mRNA levels, but decreased plasma CBG levels and hepatic CBG mRNA levels in adults (Berdusco et al., 1993). Therefore, the response of the CBG gene to glucocorticoids may be developmentally regulated.

D'agostino and Henning (1982) demonstrated that the response of CBG to thyroxine (T4) increased with advancing age during development. In the adult rat, T4 administration increased serum CBG and hepatic CBG mRNA levels, but did not influence the rate of CBG gene transcription, which was demonstrated by

nuclear run-off experiment (Smith and Hammond, 1992). Thus, the elevated hepatic CBG mRNA in these rats is probably mediated by increased CBG mRNA stability

Estrogen is also involved in the regulation of adult CBG concentrations. The administration of estrogen to nonpregnant women resulted in a significant rise in CBG concentration similar to that of noninjected pregnant women (Slaunwhite and Sandberg, 1959). The adult male rat injected with estrogen showed a 100% increase in CBG secretion using the perfused liver system (Feldman et al., 1979).

Definition of stress

A precise definition of stress has not been founded because the concept is applied to so many different phenomena. Selye (1950) first described stress as a state manifested by a specific syndrome that consists of all the nonspecifically induced changes within a biological system. Selye's four stages of the stress reaction have been described as an initial alarm reaction, a stage of resistance, a stage of adrenal hypertrophy, gastrointestinal ulceration, and thymic and lymphoid shrinkage, and a final stage of exhaustion and death (Johnson et al., 1992).

Mason (1971) found that various psychosocial stimuli could induce responses similar to those induced by Selye's physical stimuli. It has also been suggested that physical stimuli may involve an emotional or psychological

component, and that this psychological component is the more important determinant of the response (Burchfield, 1979). Therefore, interest and research in stress biology has shifted from physical causes to psychosocial influences.

Recently, stress has been defined as a complex of general and integrative responses by the central nervous system (CNS), autonomic nervous system (ANS), and HPA axis, and target organs that induce changes directed toward maintaining homeostasis (Clark et al., 1997a). In response to averse stimuli, the CNS, pituitary gland, adrenal gland, kidney, pancreas and immune system induce a number of neural, biochemical, metabolic, endocrinologic, immune, and behavioral responses (Clark et al., 1997a).

Biological responses of stress

A stress response begins with the central nervous system (CNS) perceiving a potential threat to homeostasis. Several areas in the brain are involved in the evaluation of an external event (a stressor) and organization of the biological defenses to the event (Moberg, 1985). Neurons in the hypothalamus and brain stem are sensitive to internal physicochemical stimuli and to external physical and psychosocial stimuli (Clark et al., 1997a). These areas can respond to basic and vital needs by initiating autonomic, innate, and adaptive responses. The limbic system of the CNS affects the psychological state and feeling that involve learning and memory. The cerebral cortex and

associative neurons provide responses that are not innate but are acquired by experience, such as, sight, smell, taste, and touch (Clark et al., 1997a).

Perception of a threat by the CNS results in the development of biological responses that involve behavioral, autonomic nervous system, neuroendocrine or immune responses (Moberg, 2000). For most threats, a behavioral response is the first, simplest and the most biologically cost-effective response. The animal may avoid the threat by simply removing itself from it. In the case of a mild disturbance, an animal may simply move to another location. This may involve only the CNS and voluntary muscle activity, and there may be little or no sympathoadrenal (SA) or HPA response (Moberg, 1985). Behavioral responses are not appropriate for all stressors, and an animal's behavioral options are sometimes limited or thwarted by confinement (Moberg, 2000).

If overt behavioral activities do not remove the disturbance or threat, more subtle and uncontrollable physiologic mechanisms are activated. The autonomic nervous system is activated by sudden or acute stimuli and mediated quickly (within seconds), and is responsible for the flight-or-fight response (Asterita, 1985). During stress, the autonomic nervous system affects the cardiovascular system, the gastrointestinal system, the exocrine glands and the adrenal medulla to result in changes in heart rate, blood pressure and gastrointestinal activity (Moberg, 2000). These biological changes induced by the autonomic nervous system quickly provide the energy necessary to maintain homeostasis and involve a shift of energy substrate from storage sites to the blood stream (Clark et al., 1997a).

The hypothalamic-pituitary neuroendocrine response is activated quickly but manifested more slowly than the autonomic nervous system response (Clark et al., 1997a). After a stimulus is perceived by the brain, corticotrophin-releasing hormone (CRH) is released from the hypothalamus. This hormone stimulates the anterior pituitary gland to release adrenocorticotrophic hormone (ACTH), which in turn stimulates the adrenal gland to release glucocorticoids (Saffran et al., 1955). In averse situations, there are increases in secretion of CRH within a few seconds, of ACTH within perhaps 15 seconds, and of glucocorticoids within a few minutes (Clark et al., 1997a). Various stimuli cause different patterns of release of these hormones by the hypothalamus.

Immune response can be modulated by other stress-responsive systems, especially the HPA axis (Blecha, 2000). Stress-mediated increase in glucocorticoids generally exerts immunosuppressive and anti-inflammatory effects. These effects include reductions in lymphatic tissue mass, decrease in the number of circulating lymphocytes, increase in the neutrophilic or heterophilic granulocytic interference with cell-mediated immunity, and enhancement of suppressor T-cell function (Clark et al., 1997b). Noxious stimuli also activate macrophages to release cytokines, which are a group of regulatory molecules, variously called lymphokines, interleukins, and interferons.

Measurement of the stress response

A variety of behavioral, autonomic nervous system, endocrine and immunological end-points have been used to measure stress (Moberg, 2000). An animal's abnormal behavior has been used as an indicator of stress and this depends on many factors, such as species of animal, individual characteristics, previous experience, nature and severity of stimulus, motivation, presence of deterrents, and opportunity to cope with its situation (Clark et al., 1997b). Specific behavioral signs that may provide clues to stress include levels of activity, posture, vocalization, temperament, locomotion, food and water intake, and sleep patterns (Dallaire, 1993; Hicks et al., 1998; Desautes et al., 1999; Worobec et al., 1999). Some behavioral responses can be quantified by means of observational techniques that measure the timespan of responses to stimuli, the degree of aversion and escape attempt, and behavior after the end of a stressful period (McGlone, 1985).

Determination of circulating levels of stress hormones such as catecholamines and corticosteroids has been used as an indicator of a stress response. Catecholamine concentrations (epinephrine and norepinephrine) may increase in pleasant as well as averse situations and can be measured in blood and urine samples (Asterira, 1985). Peripheral concentration of cortisol has been typically used as an indicator of stress in the pig (Becker et al., 1985). Plasma concentration of cortisol has been usually measured in blood samples taken from indwelling cannulas (Becker et al., 1985) or by jugular vein puncture (Hicks et al.,

1998) in the pig. However, cortisol half-life, pool size and volume of distribution in circulation are determined largely by the concentration of CBG, and total plasma cortisol concentrations cannot be related to cortisol production rate (Bright, 1995). It has been suggested that both the binding capacity of CBG and free cortisol concentration should be considered to assess adrenal response to social stress in Standardbred or Thoroughbred horses aged 4 to 20 years (Alexander and Irvine, 1998).

It has been demonstrated that acute stress induces a significant decrease in the number and percentage of lymphocytes and monocytes, and an increase in numbers and percentages of neutrophils in the blood(Dhabhar et al., 1995). Absolute numbers and relative proportions of blood leukocytes provide an important representation of the state of activation of the immune system (Dhabhar et al., 1996). Changes in leukocyte distribution have been used to assess the stress response in growing pigs (McGlone et al., 1993), prepubertal pigs (Morrow-Tesch et al., 1994) or gilts (Hicks et al., 1998).

Serum haptoglobin levels have been measured to indicate a potential stress response (Francisco et al., 1996; Hicks et al., 1998). Cortisone administration in rats increased serum haptoglobin concentration (Krauss, 1968). In humans, plasma haptoglobin concentration increased within six hours following inflammation or infection, increasing 10 to 1000 fold within 2 or 3 days (Eurell et al., 1992). The increase in plasma haptoglobin concentration following synthesis in the liver may be mediated by a endogenous leukocyte mediator which is released from stimulated monocytes during an inflammatory stress

(Klasing, 1985). Elevation of plasma haptoglobin levels is generally nonspecific regarding the nature of the inducing agent, and can be used to gauge the extent of many different pathological processes (Eurell et al., 1992; Francisco et al., 1996b)

Effects of stress on plasma CBG levels

The type and duration of stress may influence plasma CBG levels. Bassett (1986) found short -term increases in the corticosteroid binding activity of plasma after short-term acute stress. Pigs subjected to 5 hour-transport stress had higher cortisol and CBG concentration than untransported pigs (Nyberg et al., 1988).

Tinnikov (1993) reported that acute stress with ether treatment did not affect plasma CBG levels, but 6-hour restriction and 1-day starvation decreased plasma CBG levels in rats. The author suggested that the duration of stress stimulus is essential for an involvement of CBG in adaptive reactions. In pigs, surgery under anesthesia increased plasma cortisol level on the day of surgery but decreased plasma CBG levels on the day after surgery (Dalin et al., 1993) indicating that the decrease in the CBG level was not due to an increased cortisol level but to some other surgery effects such as inflammation.

Fleshner and coworkers (1995) reported that exposure of adult male rat to a single acute 90-minute session of 80 - 100 inescapable tail shocks (IS) resulted in an increase in basal total serum corticosterone and decrease in plasma CBG

levels for 24 - 48 hours after IS termination. The authors suggested that the IS-induced CBG decrease could be due to an increase in clearance or a decrease in CBG synthesis rather than a decrease in binding affinity to corticosterone.

In the rat, a 2-week chronic social stress produced a decrease in plasma CBG levels. A larger decrease in CBG levels was observed in subordinate rats (nearly 70%) than in dominant rats (40%) (Spencer et al., 1996). In this study, subordinate rats were subjected to significant weight loss and wounding. The authors suggested that the reduction in CBG levels might be due to increased degradation of CBG by increased neutrophil elastase activity resulted from the inflammation response to wounding rather than a change in CBG protein production.

Tannenbaum and coworkers (1997) examined changes in plasma CBG following acute stress in adult rats. Restraint stress produced a significant decrease in plasma CBG, and elevation in basal ACTH and corticosterone 24 hours later. However, animals exposed to restraint stress during the dark phase of the diurnal cycle showed no change in plasma CBG binding despite the presence of high absolute plasma corticosterone levels. Alexander and Irvine (1998) investigated the effect of social instability on plasma CBG, total and free cortisol levels in horses. CBG binding capacity was lower and free cortisol higher in newcomers than in residents, but total cortisol did not differ between the groups. The authors suggested that it is essential to monitor the CBG binding capacity and free cortisol concentrations in addition to total cortisol levels for assessment of adrenal axis status accurately in horses.

The influence of different lengths and intensities of stressful situations on circulating levels of CBG was investigated in adult male rats (Marti et al., 1997). In this study, short-term exposure (e.g., 1 hour) to severe stressors such as immobilization (IMO) did not change serum CBG level, but 6-hour or 24-hour IMO significantly decreased serum CBG levels. A reduction of CBG levels was not observed by exposing the rats to 24 hours of food and water deprivation.

Changes in plasma CBG levels during acute stress were examined in 4-week-old pigs (Hicks et al., 1998). Heat, cold, or shipping treatment for 4 hours did not affect the plasma CBG levels but had an effect on plasma cortisol levels.

Therefore, these data indicate that the conditions of stress exposure and the ability to control stress should be considered to fully understand the influence of stress on plasma CBG levels.

Significance of the stress-induced reduction in plasma CBG levels

The function of the stress-induced reduction in plasma CBG levels is unclear, but there are several theories. A reduction in plasma CBG levels may serve to increase free cortisol, thereby producing greater stimulation of target tissues to combat stress (Fleshner et a., 1995). Although the free fraction of plasma cortisol increases when plasma CBG level decreases, the absolute concentration of free cortisol will not increase unless the cortisol secretion rate accelerates. This is because cortisol half-life, pool size, and volume of distribution are inversely proportional to plasma CBG levels (Bright, 1995).

Under resting conditions, an increase in free cortisol concentration has a negative feedback on the HPA axis, reducing activity until free cortisol level returns to its set point. However, an increase in plasma CBG level with an increase in plasma cortisol level keeps free cortisol concentration within normal range (Norman and Litwack, 1997).

A second theory is that the reduction in plasma CBG level reflects the targeted release of cortisol at selected sites. CBG is cleaved by neutrophil elastase and undergoes a conformational change that disrupts its steroid-binding activity (Pemberton et al., 1988). The cleavage of CBG by neutrophil elastase results in the release of cortisol directly to the activated neutrophil (Hammond et al., 1990a). Therefore, the cleavage of CBG by neutrophil elastase may provide a mechanism for the direct delivery of relatively large amounts of cortisol to the activated neutrophils at the sites of inflammation (Hammond et al., 1990b).

A third theory is that CBG itself may serve as a hormone (Rosner et al., 1987). There is evidence that CBG binds to the plasma membranes and is internalized via its binding site (Kuhn, 1988). The interaction of CBG with target cell and plasma membrane provides the active delivery of cortisol to the target cells (Rosner, 1990) or activation of a second messenger system (Rosner et al., 1987). Therefore, CBG appears to serve as a hormone, and CBG receptor stimulation in target tissues may have an important role under condition of stress.

CHAPTER 2. DEVELOPMENT OF A PORCINE CORTICOSTEROID-BINDING GLOBULIN (CBG) cDNA PROBE AND AN INVESTIGATION OFCBG EXPRESSION SITES IN THE POSTNATAL PIG

Abstract

The purpose of this study was to develop a porcine CBG cDNA probe in order to examine the porcine CBG mRNA expression in major tissues from the postnatal pig. The reverse transcriptase-polymerase chain reaction (RT-PCR) was conducted to develop the porcine CBG cDNA probe from liver total RNA extracted from pigs on 40 days of age. The RT-PCR product was subcloned into the pGEM vector (Promega, Madison, WI) and subjected to restriction enzyme treatment and DNA sequencing. Northern blot analysis was conducted using total RNA extracted from samples (~ 200 mg) of liver, lung, kidney and whole adrenal tissue that were collected from pigs on day 3 (n = 2) or 40 (n = 2) of ages. A 500 base pair (bp) partial porcine CBG cDNA encoded 166 amino acids and had 83%, 78%, and 77% homology to a 494 nucleotide sequence of sheep, human, and rabbit, respectively. The deduced peptide sequence of the partial porcine CBG showed 77%, 62%, 60% and 51% homology to sheep, human, and rabbit, and rat CBG sequences, respectively. An approximately 1.53-kilobase CBG mRNA was detected only in the liver tissue. In conclusion, the

development of a partial CBG cDNA for swine makes it possible to study the ontogeny and the regulation of CBG synthesis at the molecular level, and the liver is the primary source of CBG biosynthesis in the postnatal pig.

Introduction

Glucocorticoids, primarily cortisol in the pig, play an important role in preparing the fetus for birth (Liggins, 1994), induction of parturition (First and Bosc, 1979), postnatal development of various organ systems (Henning, 1981), and adaptation to stress (Munck et al., 1984). Glucocorticoids in circulation exist in both free and protein-bound forms (Ballard, 1979).

Corticosteroid-binding globulin (CBG) is the major transport protein for glucocorticoids within the circulation of the majority of animals. CBG is characterized as a 50-60 kD monomeric glycoprotein with a single steroid binding site that interacts with biologically active glucocorticoids (Mickelson et al., 1982). The primary structure of human CBG was deduced from the cDNA sequence of CBG isolated from human liver and lung cDNA libraries (Hammond et al., 1987). Human CBG contains 383 amino acids and its' calculated polypeptide molecular weight is 42, 646 (Hammond et al., 1987). The availability of a human CBG cDNA has facilitated the cloning of CBG cDNA and characterization of the primary structure of CBG from several other species (Smith and Hammond, 1989; Seralini et al., 1990). Even though there is a low degree of sequence similarity between CBG from different species, phylogenetic comparison of their

primary structure shows a highly conserved region that may be structurally and/or functionally important (Hammond, 1990).

The identification of tissue sites responsible for biosynthesis of CBG has been studied in a number of animals using species-specific CBG cDNA. The liver has been identified as the major site of CBG biosynthesis in most species examined (Khan et al., 1984; Smith and Hammond, 1989; Serlini et al., 1990; Jacobs et al., 1991). However, CBG mRNA expression has also been demonstrated in the kidney and testis of the rhesus monkey (Hammond et al., 1987), human lung (Hammond et al., 1987) and placenta (Misao et al., 1999), and in the spleen and ovary of the adult rabbit (Seralini et al., 1990). CBG biosynthesis by tissues other than the liver may influence the local bioavailability of cortisol, which, in turn, may affect fetal maturational events that are essential for normal development (Scrocchi et al., 1993).

This study was conducted to develop and characterize porcine CBG cDNA, and to examine the porcine CBG mRNA expression in major tissues of the postnatal pig. The partial porcine CBG cDNA was also characterized by comparison with CBG cDNA's from other species.

Materials and methods

Materials. A First-strand synthesis kit (RETROscript) for reverse transcription was purchased from Ambion (Austin, TX). AmpliTag polymerase and supplements for PCR amplification were purchased from Perkin Elmer

(Foster City, CA). The designed primer set was purchased from Integrated DNA Technologies, Inc. (Coralville, IA). pGEM-T easy vector and restriction enzymes were purchased from Promega (Madison, WI). Epicurian Coli XL1-Blue supercompetent cells and a random primer labeling kit was purchased from Stratagene (La Jolla, CA). The ABI prism Dye Terminator Cycle Sequencing reaction kit was purchased from Perkin-Elmer Inc. (Foster City, CA). The Tri-Reagent, yeast total RNA, and Sephadex G-50 were purchased from Sigma Chemical (St Louis, MO). Nylon transfer membranes were purchased from Schicher & Schuell (Keene, NH). The [α-^{32}P] dCTP was purchased from Du Pont NEN (Boston, MA). Kodak X-Omat film was purchased from Eastman Kodak (Rochester, NY). Human CBG cDNA was donated by Dr. G. L. Hammond[1]. All other chemicals were purchased from Sigma Chemical (St Louis, MO) and Fisher Scientific (Pittsburgh, PA).

Animals. Intact male pigs (n=4) of Landrace, Duroc and Hampshire breeding were used on day 3 or 40 following birth. All pigs were provided 20% crude protein-pelleted creep feed beginning on day 14 of age and weaned on day 24 following birth. Food and water were provided ad libitum. On either day 3 (n = 2) or 40 (n = 2) of age, pigs were placed under general anesthesia using halothane and samples (~200 mg) of liver, lung, kidney, and whole adrenal tissue were collected. Animal use in this study was approved by the University of Tennessee Animal Care and Use Committee.

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RNA Extraction. Total RNA was extracted from freshly collected pig liver, lung, kidney and adrenal tissues with Tri-Reagent using a slight modification of the standard procedure (Sambrook et al., 1989; see Appendix A). Total RNA was quantified spectrophotometrically at 260 nm and stored at –80°C. The RNA was electrophoresed in a 1.3 % agarose gel containing formaldehyde, and stained with ethidium bromide to determine the integrity of the total RNA.

Development of Porcine CBG cDNA. A partial porcine CBG cDNA was developed from porcine liver total RNA using the reverse transcriptase-polymerase chain reaction (RT-PCR) (Sambrook et al., 1989). Internal sequences homologous among sheep and human CBG cDNA were used to design primers for porcine CBG [2]. The primer set consisted of the forward primer (5'-GTGAAGGTGCCCATG ATGTT-3'OH) and the reverse primer (5'CAGGTGAAGTSGTCGAAGA-3'OH) that allowed amplification of a cDNA sequence coding from base 720 to 1213 relevant to human CBG cDNA (Hammond et al., 1987).

Reverse transcription (RT) used to produce the first single-strand DNA was performed using total RNA isolated from pig liver. Briefly, 2 μg of total RNA, 2.5 mM of each dNTP mixture, and 5uM of random decamers were mixed, heated for 3 minutes at 80°C, and then placed on ice. 1x RT-PCR buffer (100 mM Tris-HCl, 500mM KCl, 15mM $MgCl_2$, pH 8.3), 0.5 Units of placental Rnase

inhibitor, and 5 Units of M-MLV Reverse Transcriptase were added to the mixture to the final volume of 20 μl and incubated at 43°C for 1 hour. The mixture was incubated at 92°C for 10 minutes to inactivate the Reverse Transcriptase, and stored at –20°C for further processing.

In the amplification step, 5 μl of the RT reaction, 1x reaction buffer (100mM Tris-HCl, 100mM KCl, pH 8.3), 200 μM of each dNTP, 1 μM of each primer, and 10 U/50 μl of AmpliTag DNA polymerase (Stoffel Fragment) and 4mM of $MgCl_2$ were mixed to final volume of 50 μl. Human CBG cDNA was used as a positive control. The reaction parameters were 2 minutes at 95°C for 1 cycle in the initial step, 30 seconds at 95°C, 60 seconds at 51°C and 45 seconds at 72°C for 35 cycles in the cycling step, and then 5 minutes at 72°C in the final step.

Following PCR amplification, the PCR products were subsequently inserted into pGEM-T easy vectors. The recombinant plasmids were used to transform Epicurian Coli XL1-Blue supercompetent cells. The transformants were propagated in Luria-Bertani (LB) broth in the presence of ampicillin to amplify the plasmids. Plasmids were isolated by the alkaline lysis method (Sambrook et al., 1989) and digested with restriction enzymes EcoR I and Ras I to confirm the correct size of the PCR products.

The PCR products were subjected to DNA sequencing for confirmation. DNA sequencing was performed with the ABI prism Dye Terminator Cycle

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[2] Assistance was provided by Dr. R. L. Matteri, Animal Physiology Research Unit, USDA-ARS, Colombia, MO, in the design of the primers.

Sequencing reaction kit on an ABI 373 DNA sequencer (Perkin-Elmer Inc., Foster City, CA). The initial sequence data text files were edited following comparison with the same data displayed in four-color electropherograms before they were analyzed further.

Northern Blot Analysis. The Northern blot procedure for determining the expression of CBG mRNA was modified from that of Sambrook et al.(1989) as outlined in Appendix B. Autoradiography was carried out at –70°C using Kodak X-Omat film to detect the radioactivity exposed from the radiolabeled porcine CBG probe.

Results

A 500 bp partial porcine CBG cDNA was obtained from pig liver total RNA using RT-PCR (Figure 2-1). The size of the partial porcine CBG cDNA was close to the PCR product obtained from a human CBG cDNA template used as a positive control. After the partial porcine CBG cDNA was inserted into a pGEM-T easy vector, digestion of the recombinant plasmid with restriction enzyme EcoR I produced the 3000 bp vector fragment and the 518 bp insert fragment (Figure 2-2). The digestion of the insert fragment with restriction enzyme Rsa I produced a 304 bp fragment and a 174 bp fragment at the expected restriction site (Figure 2-3).

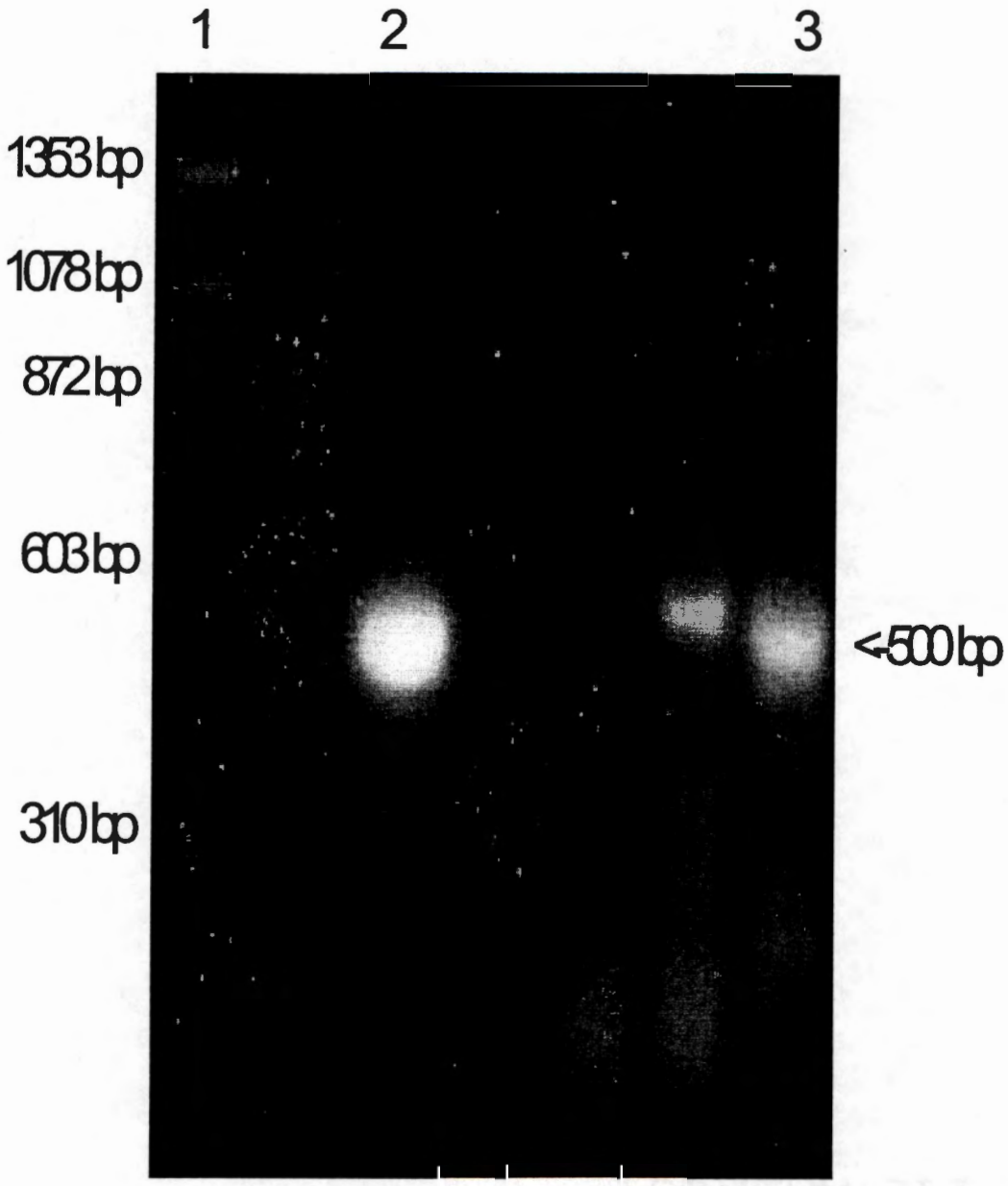


Figure 2-1. RT-PCR analysis of pig liver total RNA for CBG. Lane 1, ΦX174 DNA/HaeIII marker; lane 2, human CBG cDNA as a positive control template; lane 3, pig cDNAs synthesized from liver total RNA.

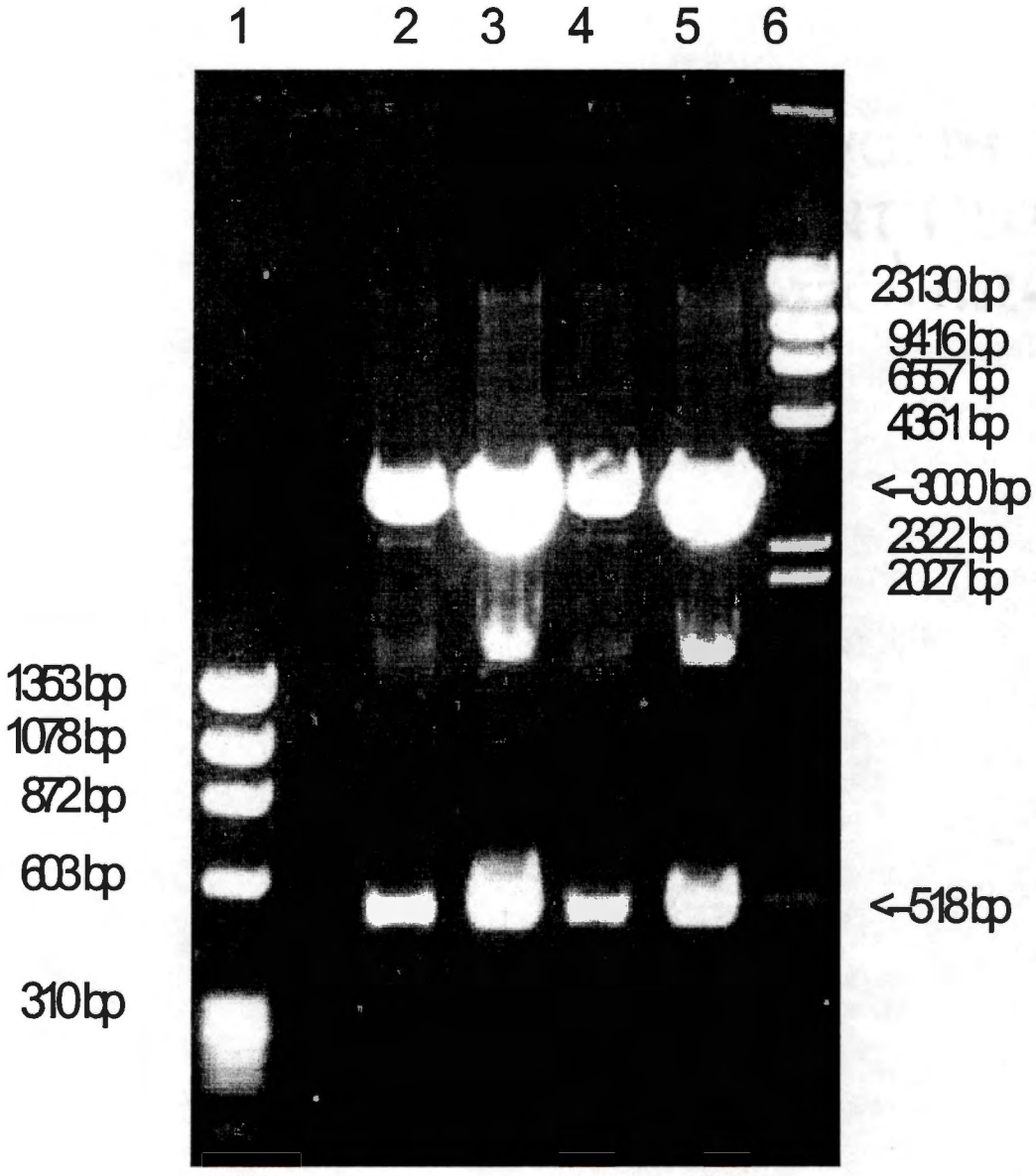


Figure 2-2. Digestion of the recombinant plasmid containing PCR product with EcoR I restriction enzyme. Lane 1, ΦX174 DNA/HaeIII marker; lane 2, 3, 4, 5, the recombinant plasmid digested with EcoR I; lane 6, λDNA/Hind III marker.The arrows indicate the vector fragment (3,000 bp) and the insert fragment (518 bp).

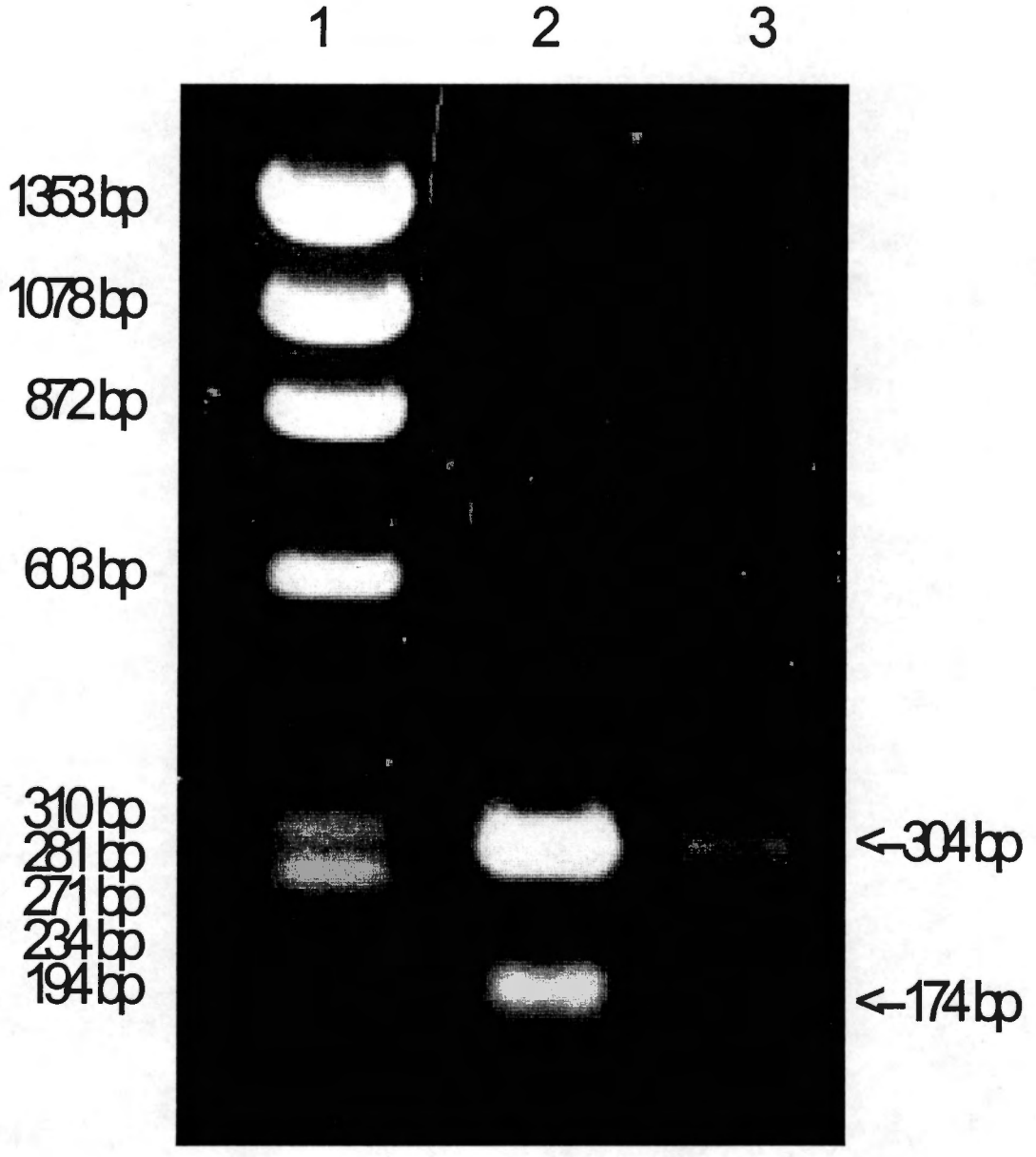


Figure 2-3. Digestion of the PCR product with Rsa I restriction enzyme. Lane 1, ΦX174 DNA/HaeIII marker; lane 2 and 3, The digested fragments of PCR product with Rsa I.

Nucleotide sequence analysis revealed that the partial porcine CBG cDNA encoded 166 amino acids (Figure 2-4). The deduced primary structure of the partial porcine CBG cDNA showed one cysteine residue and three asparagine residues. The partial porcine CBG cDNA sequence was located between the 720 and 1219 bp with respect to the human CBG cDNA sequence. The deduced primary structure of the partial porcine CBG cDNA was located between amino acids 207 and 370 with respect to human CBG primary structure.

The sequence of the partial porcine CBG cDNA was compared with published sequences from that of the human, sheep, rabbit, and rat (Figure 2-5). The partial porcine CBG cDNA was found to contain six more bases than that recorded for CBG from human, sheep and rabbit, and nine more bases than that found in rat CBG. The partial porcine CBG cDNA had an 83%, 78% and 77% homology to a 494 nucleotide of sheep, human and rabbit CBG, respectively. Homology with a 491 nucleotide of rat CBG was low (61%). The deduced peptide sequence of the partial porcine CBG was also compared with published peptide sequences of other species. The partial porcine CBG contained two more amino acids (threonine and arginine) than sheep, human and rabbit CBG, and three more amino acids (threonine, arginine and valine) than that of rat CBG (Figure 2-6). The peptide sequence of the partial porcine CBG showed 77%, 62%, 60%, and 51% homology to sheep, human, rabbit, and rat CBG sequences, respectively.

When total RNA from 40 day-old porcine liver was analyzed by Northern blotting, an approximate 1.53-kilobase CBG mRNA was detected following 24

```
720  GTG AAG GTG CCC ATG ATG TTC CAG TCG CGC GCC ATG AAG TAC
207   V   K   V   P   M   M   F   Q   S   R   A   M   K   Y

762  TTG AAT GAC TCC TTG CTC CCC TGC CAG CTG GTG CAG CTG GAA
221   L   N   D   S   L   L   P   C   Q   L   V   Q   L   E

804  TAC ACG GGC AAT GAG ACG GCC TTC TTC ATC CTC CCG GTC AAG
235   Y   T   G   N   E   T   A   F   F   I   L   P   V   K

846  GGG GAG ATG GAC ACG GTC ATT GCC GGG CTG AGC CGG GAC ACC
249   G   E   M   D   T   V   I   A   G   L   S   R   D   T

888  ATT CAG AGG TGG TCG AAG TCC CTG ATC CCC AGC CAG GTG GAC
263   I   Q   R   W   S   K   S   L   I   P   S   Q   V   D

930  CTG TAC GTC CCA AAG GTC TCC ATC TCC AGA GCC TAT GAC CTC
277   L   Y   V   P   K   V   S   I   S   R   A   Y   D   L

972  GGG AGC ATC CTG GGG GAC ATG GGC ATT GTG GAC TTG CTC AGC
291   G   S   I   L   G   D   M   G   I   V   D   L   L   S

1014  CAC CCA ACA CAC TTC TCA GGC ATC ACC CAG AAT GCC CTG CCG
 305   H   P   T   H   F   S   G   I   T   Q   N   A   L   P

1056  AAG ATG TCC AAG GTG GTC CAC AAG GCA GTT CTG CAA TTT GAC
 319   K   M   S   K   V   V   H   K   A   V   L   Q   F   D

1098  GAG AAG GGC ATG GAG GCA GCT GCC CCC ACT ACG CGT GGA CGC
 333   E   K   G   M   E   A   A   A   P   T   T   R   G   R

1140  AGC CTG CAC GCG GCG CCC AAG CCT GTC ACT GTC CAC TTC AAC
 347   S   L   H   A   A   P   K   P   V   T   V   H   F   N

1182  CGG CCC TTC ATC GTC ATG GTC TTC GAC CAC TTC ACC TG
 361   R   P   F   I   V   M   V   F   D   H   F   T
```

Figure 2-4. Nucleotide sequence of a partial porcine CBG cDNA and deduced amino acid sequence of the partial CBG. The starting numbers for nucleotide sequence and amino acid sequence are based on those for nucleotide sequence of human CBG cDNA and for amino acid sequence of human CBG (Hammond et al., 1987).

	720			730			740			750			
porcine	GTG	AAG	GTG	CCC	ATG	ATG	TTC	CAG	TCG	CGC	GCC	ATG	AAG
human	---	---	---	---	---	---	--G	---	---	A--	A--	--C	-GT
sheep	---	TG-	---	---	---	---	---	---	---	AA-	A--	--C	---
rabbit	---	-T-	---	---	---	---	---	---	---	A--	A-T	G-C	---
rat	---	---	---	---	---	---	G--	---	--A	G--	AG-	--T	GGT

	760			770			780			790			
porcine	TAC	TTG	AAT	GAC	TCC	TTG	CTC	CCC	TGC	CAG	CTG	GTG	CAG
human	---	C-T	C--	---	--A	GA-	---	---	---	---	---	---	---
sheep	---	C--	--C	---	--G	G--	---	---	---	---	---	--A	---
rabbit	---	C--	C-C	---	C-G	G--	---	---	---	-G-	---	---	---
rat	---	--T	CG-	---	--A	G-C	T--	---	---	---	A--	A-A	---

	800				810			820			830		
porcine	CTG	GAA	TAC	ACG	GGC	AAT	GAG	ACG	GCC	TTC	TTC	ATC	CTC
human	A--	A-C	---	GT-	---	---	-G-	--T	-T-	---	---	---	--T
sheep	---	--C	--T	--A	---	---	---	--T	-T-	---	---	G-G	---
rabbit	---	--T	---	GT-	---	---	-GC	--C	--G	---	---	---	---
rat	A--	--C	--T	GT-	--A	---	-GA	--T	---	---	---	--T	--T

	840				850			860			870		
porcine	CCG	GTC	AAG	GGG	GAG	ATG	GAC	ACG	GTC	ATT	GCC	GGG	CTG
human	---	-A-	---	---	A--	---	A--	--A	---	--C	--T	-CA	---
sheep	--A	---	---	---	A--	---	---	T--	---	--C	A-T	-C-	---
rabbit	---	-A-	---	---	A--	G--	---	---	---	--C	---	-CC	---
rat	--A	-A-	---	--C	C--	---	---	--T	---	--C	--T	-CA	--T

	880				890			900			910		
porcine	AGC	CGG	GAC	ACC	ATT	CAG	AGG	TGG	TCG	AAG	TCC	CTG	ATC
human	---	---	---	--G	---	A-C	---	---	--C	GCA	GG-	---	-C-
sheep	---	---	---	---	---	---	---	---	--C	---	---	--A	-C-
rabbit	---	A--	---	--G	---	---	C-C	---	--C	--A	---	---	-C-
rat	--T	---	---	--A	---	G-T	---	---	GGC	---	CTT	A--	-C-

	920				930			940			950		
porcine	CCC	AGC	CAG	GTG	GAC	CTG	TAC	GTC	CCA	AAG	GTC	TCC	ATC
human	AG-	---	---	---	---	---	---	A-T	---	---	---	A--	---
sheep	ATG	---	---	---	--T	---	---	A--	---	---	A--	---	---
rabbit	TA-	--G	-TG	---	C--	---	---	A--	--G	---	-C-	---	---
rat	--A	--G	---	---	A--	--A	---	A--	--G	--A	T--	---	--G

	960				970			980				990	
porcine	TCC	AGA	GCC	TAT	GAC	CTC	GGG	AGC	ATC	CTG	GGG	GAC	ATG
human	--T	G--	-T-	---	---	--T	--A	GAT	G-G	---	-A-	--A	---
sheep	--T	G--	---	--C	---	--T	---	G--	---	A--	---	--T	---
rabbit	---	G--	---	--C	--G	---	A--	G--	GCA	---	-C-	-C-	---
rat	--T	GAT	A--	---	---	--T	AAA	GA-	G-G	---	-AA	---	C--

Figure 2-5. Comparison of a partial porcine CBG cDNA sequence with human (Hammond et al., 1987), sheep (Berdusco et al., 1993), rabbit (Seralini et al., 1990), and rat (Smith and Hammond, 1989) CBG cDNA sequences. CBG cDNA sequences are aligned with respect to the porcine CBG cDNA sequence, and identical bases in other sequences are indicated by a dash. The deleted bases are indicated by x.

	1000					1010				1020				1030
porcine	GGC	ATT	GTG	GAC	TTG	CTC	AGC	CAC	CCA	ACA	CAC	TTC	TCA	
human	---	---	-CA	---	---	T--	-C-	A--	-AG	G--	A-T	---	---	
sheep	---	---	-CA	---	---	---	---	A--	-GG	---	--T	---	---	
rabbit	---	--C	-CA	---	---	T--	-C-	A--	-AG	G--	A-T	---	---	
rat	AA-	---	AA-	---	---	---	-C-	A--	-AA	T--	G-T	---	---	

	1040					1050				1060				1070
porcine	GGC	ATC	ACC	CAG	AAT	GCC	CTG	CCG	AAG	ATG	TCC	AAG	GTG	
human	C--	---	---	---	G-C	---	-A-	-T-	---	TCA	--A	---	---	
sheep	---	---	---	--A	G-G	---	---	---	---	G--	--A	---	---	
rabbit	A--	---	T--	---	G-G	-G-	-CA	-T-	---	G-A	G-A	---	---	
rat	---	-A-	---	A-A	G--	-TT	-CC	TT-	-CA	T-A	T-A	-TG	xxx	

	1080					1090				1100				
porcine	GTC	CAC	AAG	GCA	GTT	CTG	CAA	TTT	GAC	GAG	AAG	GGC	ATG	
human	---	--T	--A	--T	--G	---	---	C-C	A-T	---	G--	--T	G--	
sheep	---	---	---	--C	-CG	---	--T	G-C	--T	---	---	---	T--	
rabbit	C--	--T	---	--T	--G	---	--G	C-C	---	---	C-C	--G	GG-	
rat	---	---	---	--C	A-G	--A	---	C-G	--T	--A	GG-	AAT	G--	

	1110			1120			1130			1140				
porcine	GAG	GCA	GCT	GCC	CCC	ACT	ACG	CGT	GGA	CGC	AGC	CTG	CAC	
human	--C	A--	---	-G-	T--	---	xxx	xxx	--G	GT-	-C-	--A	A--	
sheep	---	--G	--C	---	--T	--C	xxx	xxx	C-G	GT-	---	G--	ACG	
rabbit	-T-	-AG	-TG	--T	G--	--G	xxx	xxx	---	G--	CC-	---	--G	
rat	TT-	C-T	AA-	T-T	A--	-AC	xxx	xxx	--G	GCT	CC-	--A	---	

	1150			1160			1170			1180				
porcine	GCG	GCG	CCC	AAG	CCT	GTC	ACT	GTC	CAC	TTC	AAC	CGG	CCC	
human	CT-	A--	T--	---	---	A--	-TC	T-G	-GT	---	---	-A-	---	
sheep	--A	---	---	GG-	--G	C--	--C	C--	-G-	---	---	---	---	
rabbit	CT-	-T-	T-T	G-A	--C	C--	--C	C-G	A--	---	---	---	---	
rat	CT-	CGC	T-T	G-A	--A	C-T	GAC	A--	A-G	---	---	AA-	---	

	1190				1200			1210			1219
porcine	TTC	ATC	GTC	ATG	GTC	TTC	GAC	CAC	TTC	ACC	TG
human	---	---	A--	---	A--	---	---	---	---	---	--
sheep	---	---	A--	---	A--	---	---	G--	---	--G	--
rabbit	---	C--	A--	C--	A--	---	---	G--	---	---	--
rat	---	---	C--	C--	C--	--T	---	A-G	---	--A	--

Figure 2-5. Continued.

```
                207   210                       220 ^           *   230
Pig             V K V P M M F Q S R A M K Y L N D S L L P C Q L V Q L E Y T
Human           - - - - - - - L - - S T T S - - H - - E - - C - - - - M N - V
Sheep           - W - - - - - - - - N T I - - - N - - V - - C - - - - - D - -
Rabbit          - M - - - - - - - - S T V - - - H - P V - - C R - - - - D - V
Rat             - - - - - - - V - - G S I G - F R - - V F - C - I Q M D - V

                  ^   240                 250                 260
Pig             G N E T A F F I L P V K G E M D T V I A G L S R D T I Q R W
Human           - N G - V - - - - - D - - K - N - - - - A - - - - - - - N - -
Sheep           - N - - V - - V - - - - - - K - - S - - T A - - - - - - - - - -
Rabbit          - N G - - - - - - - D - - K V - - - - - A - - - - - - - - - -
Rat             G N G - - - - - - - - D Q - Q - - - - - - A - - - - - - - D - -

                        270                 280                 290
Pig             S K S L I P S Q V D L Y V P K V S I S R A Y D L G S I L G D
Human           - A G - T S - - - - - - I - - - T - - G V - - - - D V - E E
Sheep           - - - - T M - - - - - - I - - I - - - G - - - - - G - M - -
Rabbit          - - - - T Y R L - H - - I - - A - - - G - - E - R G A - A A
Rat             G - L M T - R - - N - - I - - F - M - D T - - - K D V - E -

                        300       ^     ^   310       ^         320
Pig             M G I V D L L S H P T H F S G I T Q N A L P K M S K V V H K
Human           - - - A - - F T N Q A N - - R - - - D - Q L - S - - - - - -
Sheep           - - - A - - - - N R - - - - - - - - - E - - - - V - - - - - -
Rabbit          - - - A - - F T N Q A N - - S - S - E G P L - V - - - L - -
Rat             L N - K - - - T N Q S D - - - N - K D V P L T L T M# - - -

                        330                 ^ 340               ^ 350
Pig             A V L Q F D E K G M E A A A P T T R G R S L H A A P K P V T
Human           - - - - L N - E - V D T - G S - # # - V T - N L T S - - I I
Sheep           - A - - V - - - - L - - - - - - - # # R V - V T - - - G - L -
Rabbit          - - - - L - - H - G V E V A A - # # - G P - Q - V S E - L -
Rat             - M - - L - - G N V L P N S T N # # - A P - - L R S E - - D

                        360                 370
Pig             V H F N R P F I V M V F D H F T
Human           L R - - Q - - - I - I - - - - -
Sheep           L R - - - - - - I - I - - D - -
Rabbit          L N - - - - - L I L I - - D - -
Rat             I K - - K - - - L L L - - K - -
```

Figure 2-6. Comparison of the amino acid sequence of a partial porcine CBG with those of human (Hammond et al., 1987), sheep (Berdusco et al., 1993), rabbit (Seralini et al., 1990), and rat (Smith and Hammond, 1989) CBG. Amino acid (single letter code) sequences are aligned with respect to the pig CBG sequence, and identical amino acids in other sequences are indicated by a dash. Consensus sites for N-glycosylation are indicated by a bold italic font and ^. The conserved cysteine residue is indicated by a bold italic font and *. The deleted amino acids are indicated by #.

hour autoradiographic exposure (Figure 2-7). Total RNA was extracted from liver, lung, kidney and adrenal tissues of pigs on d 3 and 40 following birth and analyzed to identify CBG mRNA expression sites (Figure 2-8). CBG mRNA expression was detected only in liver tissue on both days sampled. However, the radioactivity of hepatic CBG mRNA in pig liver on day 40 was more intense than that on day 3.

Discussion

Since the isolation of CBG cDNA from human liver (Hammond et al., 1987), the cloning of CBG cDNA has been conducted in several other species (Smith and Hammond, 1989; Seralini et al., 1990; Berdusco et al., 1993; Lin et al., 1990). Interspecies comparison of CBG cDNA sequences has revealed the existence of highly conserved regions within the DNA (Hammond et al., 1991). In the present study, the degenerate primers for porcine CBG developed in the present study were designed from the highly conserved region of human and sheep CBG cDNA sequences.

A 500 base pair PCR product was produced from pig liver total RNA using reverse transcriptase-polymerase chain reaction (RT-PCR) methodology. The comparison of the size of PCR product from pig cDNAs with that from human CBG cDNA, the digestion of the recombinant plasmid with EcoR I, the digestion of the insert with Rsa I, and the comparison of the EcoR I fragment nucleotide sequences with known human, sheep, and rabbit CBG cDNA

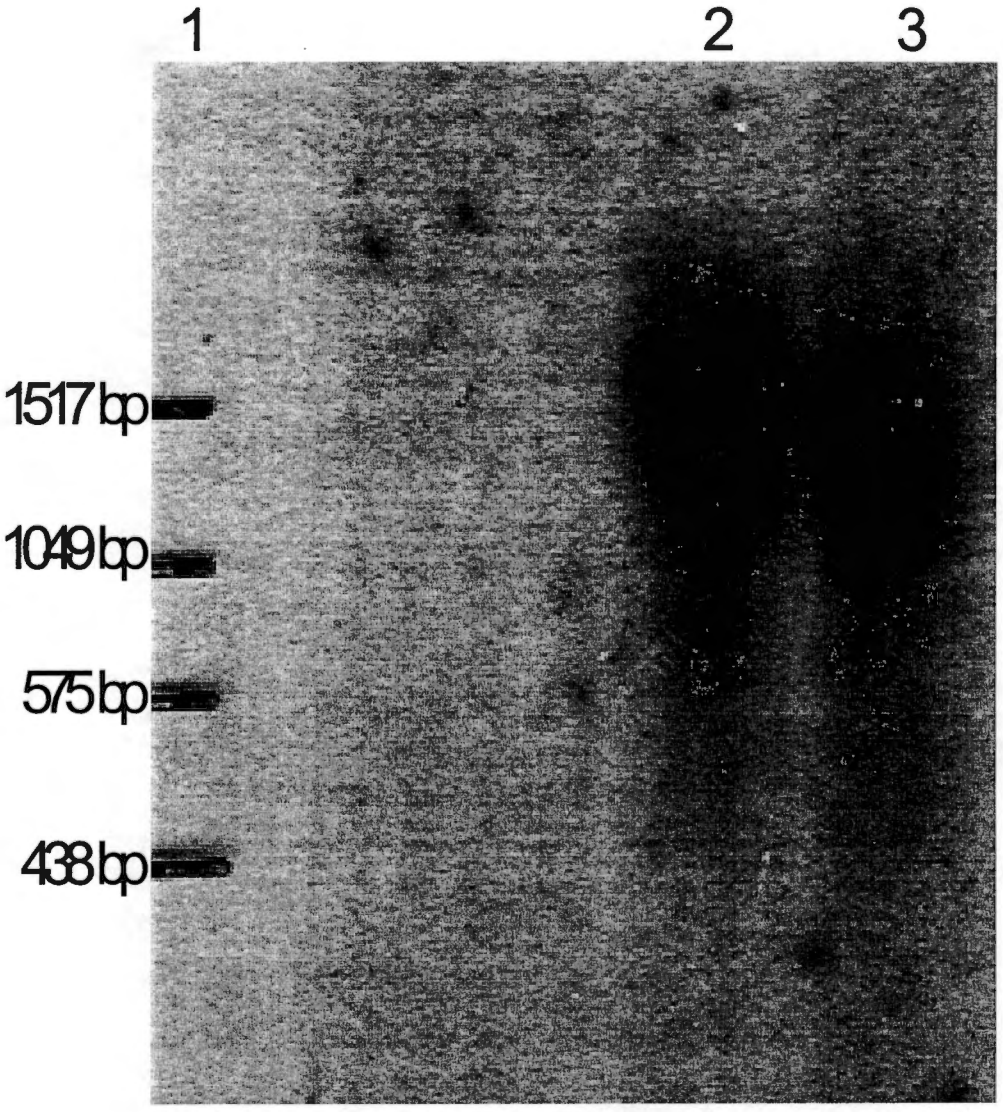


Figure 2-7. Northern blot analysis of porcine CBG expression in pig liver. Lane 1, RNA molecular weight marker; lane 2, 35 μg of pig liver total RNA; lane 3, 18 μg of pig liver total RNA.

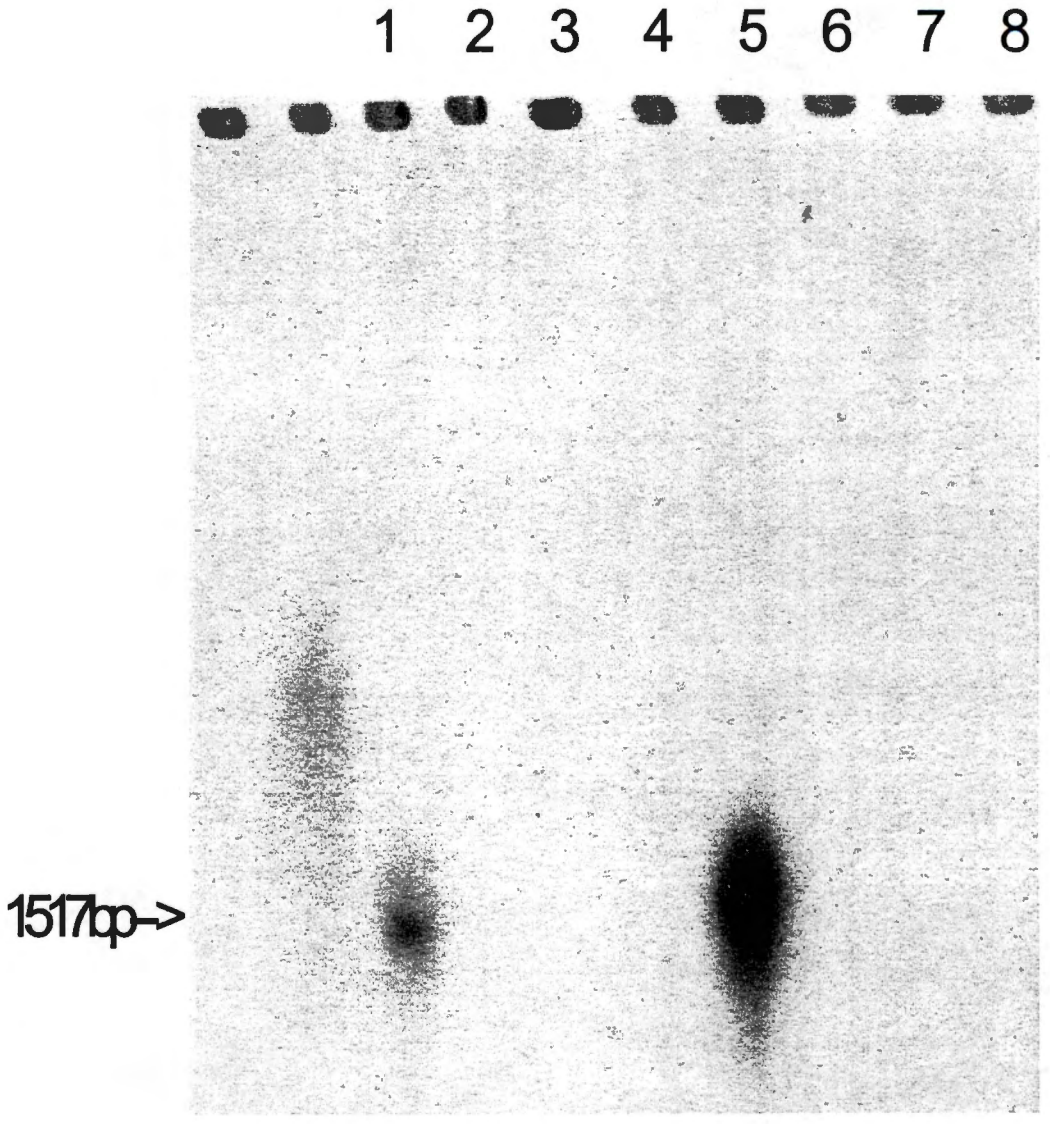


Figure 2-8. Northern blot analysis of porcine CBG expression in various tissues from pigs on day 3 and day 40. Lane 1, liver on day 3; lane 2, lung on day 3; lane 3, kidney on day 3; lane 4, adrenal gland on day 3; lane 5, liver on day 40; lane 6, lung on day 40; lane 7, kidney on day 40; lane 8, adrenal gland on day 40.

sequences confirmed that the 500 base pair PCR product produced from pig liver total RNA is a part of porcine CBG. Comparison of the primary structure of the partial porcine CBG with that of human, sheep, rabbit, and rat CBG's showed several well conserved regions. These conserved regions may have important biological function.

Affinity-labeling of human CBG with 6-bromo-progesterone demonstrated that a cysteine is located in the steroid-binding site (Khan and Rosener, 1977). A comparison of the partial porcine CBG with CBG's of human, sheep, rabbit and rat demonstrated that a cysteine residual at amino acids 228 position is located in an identical position in all five species. Furthermore, the domains containing this cysteine are highly conserved among all five species. Therefore, this region containing a cysteine might represent a functionally important domain in CBG.

When the N-glycosylation sites of human (Hammond et al., 1987), sheep (Berdusco et al., 1993), rabbit (Seralini et al., 1990), and rat CBG (Smith and Hammond, 1989) were compared to that of the partial porcine CBG, only one asparagine at the 238 position was found in an identical position in all five species. Glycosylation at this position has been shown to be an absolute requirement for steroid binding (Avvakumov et al., 1992). An asparagine at amino acids 221 position in the partial porcine CBG is also located in an identical position as ovine CBG. These consensus sites are located in highly conserved regions of the CBG. Furthermore, the consensus sites for N-glycosylation are located close to the cysteine residue in the highly conserved region, which means that this region might represent a functionally important domain in CBG.

The partial porcine CBG amino acid sequence showed relatively high homology with that of sheep (77%) in contrast to that of the rat (51%). Although the regions containing the consensus sites for N-glycosylation and a cysteine are highly conserved, the carboxyl-terminal region of CBG is very poorly conserved. This carboxyl-terminal region corresponds to the reactive region of the serine protease inhibitors that are related to CBG (Smith and Hammond 1989). Insertion of two additional amino acids was found in this region of the partial porcine CBG as compared to that in the other species examined. Rat CBG has one less amino acid in this poorly conserved region (Hammond et al., 1991). Therefore, the variation in amino acid content among species may indicate that this region is not functionally important.

The liver has been identified as the major site of CBG biosynthesis in several animal species. Additional sites of CBG biosynthesis may exist depending on species and stage of development. The availability of species-specific CBG cDNAs makes it possible to demonstrate that tissues other than the liver may also contain CBG mRNA and, therefore, possess the capacity to synthesize the protein. Hammond et al (1987) demonstrated in the rhesus monkey using RNA blot-hybridization analysis that CBG mRNA is relatively abundant in the liver, but is also present in the lung, testis, and kidney. Although CBG mRNA levels in nonhepatic tissues from fetal rabbit are very low when compared to that in liver tissue from an adult rabbit, both kidney and liver contain similar amounts of CBG mRNA in the fetus during late gestation (Serlini et al., 1990). In contrast, a comprehensive Northern blot analysis of various tissues

from the adult rat showed the presence of CBG mRNA only in the liver (Smith and Hammond 1989). In the present study, Northern blot analysis of total RNA isolated from various tissues of the pig on days 3 and 40 of age showed that only the liver expressed the CBG mRNA. The radioactivity of hepatic CBG mRNA in the 40-day old pig was relatively more intense than that of the 3-day old pig. Kattesh and Roberts (1993) found that the circulating level of CBG measured in 42-day old pigs was significantly higher than that measured in 3-day old pigs. Therefore, determination of hepatic CBG mRNA expression level in pig needs to demonstrate whether the developmental changes in hepatic CBG mRNA expression influences the circulating levels of CBG in the pig.

In conclusion, the development of a partial cDNA for swine makes it possible to study the ontogeny and the regulation of CBG synthesis at the molecular level. It appears from the results of this study that the liver is the primary source of CBG biosynthesis in the postnatal pig, and the conserved regions in CBG's primary structure might indicate important steroid-binding site of CBG conserved among species.

CHAPTER 3. RELATIONSHIPS OF PLASMA CORTISOL AND CORTICOSTEROID-BINDING GLOBULIN (CBG) CONCENTRATIONS, AND HEPATIC CBG mRNA EXPRESSION IN PRENATAL AND POSTNATAL PIGS

Abstract

The concentration of CBG in circulation is determined by its biosynthesis, peripheral degradation, and/or transfer to the extravascular system. The purpose of this study was to evaluate the relationship among hepatic CBG mRNA expression and plasma concentration of cortisol and CBG during the prenatal and postnatal periods in the pig. Blood and liver tissue were collected from fetal pigs (n = 7-14 per age) on day 50, 70, 80, 90, and 104 of gestation, as estimated by fetal crown-rump length, and from postnatal pigs (n = 8 per age) on day 1, 3, 10, 20, 30, and 40 following birth. Plasma cortisol and CBG concentrations were determined by radioimmunoassay (RIA) and an enzyme-linked immunosorbent assay (ELISA), respectively. CBG mRNA expression was determined from liver total RNA by Northern blot analysis using porcine CBG cDNA, and normalized relative to β-actin mRNA expression level. In fetal pigs, CBG mRNA expression was highest ($P < 0.01$) on day 50 as compared to day 90, exhibiting a negative relationship ($r = - 0.63$, $P < 0.01$) with estimated gestation age. Plasma CBG

concentrations were correlated ($r = 0.34$, $P < 0.05$) to CBG mRNA levels. Plasma cortisol concentrations were not different over this same period. In postnatal pigs, CBG mRNA expression increased ($P < 0.01$) from day 3 to day 40. Plasma CBG concentration increased ($P < 0.01$) from day 1 (6.1 ± 3.4 μg/ml) to day 10 (15.1 ± 3.7 μg/ml), while plasma cortisol concentration remained constant. The present study represents the first documentation of the relationships between hepatic CBG mRNA and circulating CBG concentrations in the pig. An understanding of these relationships may provide insight into the mechanisms determining the bioavailability of cortisol necessary in prenatal development and the conservation of cortisol during postnatal development.

Introduction

Adrenal glucocorticoids play an important role in fetal maturation (Sangild et al., 1994b), induction of parturition (First and Bosc, 1979), development of hepatic enzymes (Henning, 1981), and adaptation to stress following birth (Lay, 2000). Cortisol, the major glucocorticoid in swine, exists in plasma in both free and protein-bound forms (Ballard, 1979) with the free form being biologically active. Corticosteroid-binding globulin (CBG) is the major transport glyocoprotein that binds cortisol within the circulation with high affinity. CBG functions to alter the rate of cortisol disappearance (Migeon et al., 1968), and also has a pronounced influence on cortisol pool size and volume of distribution (Bright,

1995). Thus, CBG levels can regulate the plasma concentration as well as bioavailability of cortisol during development (Bright, 1995).

In most species, the concentration of cortisol in fetal circulation increases during late pregnancy, the mechanism by which this comes about varies with species (Liggins, 1994). In fetal sheep both total and free cortisol concentrations increase as a result of an increased rate of secretion (Fairclough and Liggins, 1975), whereas plasma free cortisol concentration in fetal rats increases as a result of falling concentrations of CBG (Liggins, 1994). In the fetal pig, both total and free cortisol concentration increase just before birth with a corresponding increase in CBG binding capacity (Kattesh et al., 1997), which may contribute to preparing the fetus for birth (Liggins, 1994) and induction of parturition (First and Bose, 1979). In the neonatal pig, the concentration of CBG decreases from day 3 to day 7 and increases by day 28 (Kattesh and Roberts, 1993). The increase in plasma CBG in the pig around 28 days of age reflects a corresponding shift in cortisol distribution from unbound and albumin-bound forms to a CBG-bound form (Kattesh et al., 1990).

Plasma CBG level is determined by its biosynthesis, metabolism, and/or transfer to the extravascular system. The liver appears to be the major site of CBG biosynthesis in several species including human (Khan et al., 1984), rat (Smith and Hammond, 1989), rabbit (Seralini et al., 1990), and pig (Heo et al., 2000). In the fetal rat, plasma concentrations of CBG decrease during the last third of gestation, probably due to a reduction in hepatic CBG mRNA synthesis (Smith and Hammond, 1991). This results in an increase in circulating levels of

free glucocorticoids which contribute to developmental events such as lung maturation (Hammond et al., 1991). In infant rats, plasma CBG and hepatic CBG mRNA levels were very low during the first week following birth and then increased on day 10 in concert (Elfahime et al., 1992). In contrast, hepatic CBG mRNA levels reached adult values by three weeks of age, but adult levels of plasma CBG were not attained until six weeks of age (Smith and Hammond, 1991). This might be due to a difference in clearance rate of CBG between infant and adult rats (Smith and Hammond, 1991). Plasma CBG levels during postnatal development in the rat might be determined by hepatic CBG biosynthesis as well as clearance rate of CBG in circulation (Smith and Hammond, 1991). In mares, a significant pregnancy-related decrease in plasma CBG may result from increased CBG uptake by target tissues (Martin and Silberzahn, 1990).

Therefore, determining the relationship between plasma CBG and hepatic CBG mRNA expression levels may be useful for understanding the mechanism regulating plasma CBG levels during development in pigs. This study was conducted to evaluate the relationships among hepatic CBG mRNA expression and plasma concentrations of cortisol and CBG during the prenatal and postnatal periods in the pig.

Materials and methods

Materials. Tri-Reagent, yeast total RNA, Sephadex G-50, and anti-rabbit IgG-alkaline phosphatase conjugate were purchased from Sigma Chemical (St Louis, MO). Nylon transfer membranes were purchased from Schicher & Schuell (Keene, NH). The radioisotope [α-^{32}P] dCTP was purchased from Du Pont NEN (Boston, MA). A random primer labeling kit was purchased from Stratagene (La Jolla, CA). Human β-actin cDNA control probe was purchased from Clontech (Palo Alto, CA). Kodak X-Omat film was purchased from Eastman Kodak (Rochester, NY). Coat-A-Count RIA kit was purchased from Diagnostic Products Corp. (Los Angeles, CA). ELISA 96 well plates were purchased from Corning Costar Corp. (Oneonta, NY). p-Nitrophenyl-phosphate was purchased from Kirkegard & Perry Lab. (Gaithersburg, MD). All other chemicals were purchased from Sigma (St Louis, MO) and Fisher Scientific (Pittsburgh, PA).

Animals. For the prenatal study, fetal pigs were collected from reproductive tracts removed from pregnant sows following slaughter at a local abattoir (Wampler's Farm Sausage Co., Inc., Lenoir City. TN). Fetuses were quickly removed via manual manipulation and expulsion through openings made in each uterine horn near the bifurcation and utero-tubal junction. Body weight and crown-rump length measurements were taken. Gestational age for each fetal pig was estimated using crown-rump length measures incorporated into an empirical linear regression equation [gestational age in days = (fetal length in

millimeters + 70.59) / 3.25] (Marrable and Ashdown, 1967). A total of 44 fetal pigs were collected from different sows (two litters/estimated age) on the following estimated days of gestation (number of pigs in parenthesis): day 50 ± 0.1 (n = 7), day 70 ± 0.4 (n = 7), day 80 ± 2.2 (n = 8), day 90 ± 3.0 (n = 14), and day 104 ± 1.1 (n = 8).

Forty-eight crossbred pigs (Landrace, Duroc and Hampshire breeding), born naturally and reared conventionally, were used for the postnatal study. Pigs were weaned on day 23 or day 24 following birth. All pigs were provided 20% crude protein-pelleted creep feed beginning on day 14 of age and water was provided ad-libitum. Eight pigs each were randomly allotted for study on days 1, 3, 10, 20, 30, and 40 following birth. Animal use in this study was approved by the University of Tennessee Animal Care and Use Committee.

Plasma and liver tissue collection. A single blood sample (1~ 2 ml) was taken from each fetus via cardiac puncture upon its removal from the uterus, and the sample placed in a microcentrifuge tube containing a drop of sodium heparin (1,000 Units/ml). Liver (~200 mg) was removed from each fetus following its death and quickly processed to extract total RNA as described below.

At prescribed days following birth, animals were individually weighed and pre-anesthetized with a solution of 30 parts Ketamine (100 mg/ml) and one part Acepromazine (10 mg/ml) administered intramuscularly at a dose of 0.2 ml/kg body weight. The pig was moved to the surgery room and placed under general anesthesia using closed circuit halothane administered by a gas inhalation

machine. A single blood sample (10 ml) was collected from the anterior vena cava and placed in a vacutainer tube containing a 2% solution of ethylenediaminetetraacetate (EDTA) and gently mixed. Plasma was collected by centrifugation at 2,000 x g for 10 minutes and stored at - 20°C for later analysis of cortisol and CBG. Liver (~ 200 mg) tissue was removed from the anesthetized pig and processed to extract total RNA as described below. All animals succumbed while under general anesthesia as a result of fulminating anemic anoxia.

RNA extraction. Total RNA was extracted from liver tissue with Tri-Reagent as described previously (Chapter 2; Appendix A).

Northern Blot Analysis. The Northern blot procedure for determining the expression of CBG mRNA was modified from that of Sambrook et al.(1989) as outlined in Appendix B. A 500 bp partial porcine CBG cDNA developed earlier (Chapter 2) was used as a probe to detect CBG mRNA expression in liver tissue. The expression levels of hepatic CBG mRNA were expressed relative to β-actin mRNA expression levels.

Plasma cortisol concentration. Plasma cortisol concentrations were determined using a radioimmunoassay (RIA) kit (Coat-A-Count, Diagnostic Products Corp., Los Angeles, CA) as outlined in Appendix C. Cortisol

concentration was expressed as nanogram per milliliter (ng/ml). Intra-and inter-assay coefficient of variation was 10.3 % and 7.4 % respectively.

Plasma CBG concentration. The amount of CBG in plasma was measured by a indirect enzyme-linked immunosorbent assay (ELISA) for porcine CBG (pCBG) developed by Kattesh and Roberts (1993) as outlined in Appendix D. Inter-assay and intra-assay coefficient of variation was 14.7 % and 12.4 %, respectively.

Statistics. Data were analyzed as a randomized block design using PROC MIXED procedure of SAS (SAS, 1999). Litter and replicate were included in the analysis model as random effects. The fixed effect of age was used to analyze for differences in plasma cortisol, hepatic CBG mRNA, and plasma CBG levels. Data were represented as least squares means with standard errors. Significant mean differences were separated using Fisher Least Significant Difference. Pearson correlation coefficients (Norman and Streiner, 1994) were used to determine relationships among the variables.

Results

Crown-rump length and body weight. Crown-rump length and body weight of fetal pigs are summarized in Table 3-1. Body weight of fetal pigs increased ($P < 0.01$) with gestational age, but the greatest increase in body weight occurred

Table 3-1. Crown-rump length and body weight of fetal pigs from 50 to 104 days of gestation estimated using following formula: gestational age in days = (fetal length in millimeters + 70.59) / 3.25 (Marrable and Ashdown, 1967). Table values are least squares means ± SE.

Estimated gestational age (days)	50	70	80	90	104
Number of animal	7	7	8	14	8
Crown-rump length (mm)	91.3 ± 5.1^{a}	160.7 ± 3.9^{b}	191.7 ± 3.4^{c}	226.6 ± 2.5^{d}	277.3 ± 3.3^{e}
Body weight (g)	42.2 ± 38.9^{a}	219.4 ± 30.1^{b}	354.5 ± 26.3^{c}	602.4 ± 19.3^{d}	1462.9 ± 25.5^{e}

^{a,b,c,d,e} Means within a row with uncommon superscripts differ ($P < 0.01$).

from day 90 to 104 of gestation. Body weight was not different for neonatal pigs between day 1 and day 3 (Table 3-2). Pig body weight showed the smallest increase (0.26 kg/d; $P < 0.01$) from day 20 to day 30 and the greatest increase (0.40 kg/d; $P < 0.01$) from day 30 to 40 of age.

Plasma cortisol concentration. Fetal plasma cortisol concentration was similar ($P > 0.1$) on days 50, 70, 80, and 90, but increased two- fold ($P < 0.01$) between day 90 (4.7 ± 1.1 ng/ml) and day 104 (9.1 ± 1.1 ng/ml) of gestation (Figure 3-1). Plasma cortisol concentration measured on day 1 following birth was 10-fold higher than that on day 104 of gestation (90.2 ± 8.8 vs 9.1 ± 1.1 ng/ml). There was no difference ($P > 0.1$) in plasma cortisol concentration between pigs during the postnatal sampling days studied (Figure 3-2).

Plasma CBG concentration. Plasma CBG concentration was lower ($P < 0.01$) among pigs sampled on day 90 (17. 0 ± 4.0 μg/ml) and 104 (17.0 ± 7.0 μg/ml) as compared those measured on day 50 (40 ± 11.0 μg/ml) of gestation (Figure 3-3). In postnatal pigs, plasma CBG concentration increased ($P < 0.01$) from day 1 (6.1 ± 3.4 μg/ml) to day 10 (15.1 ± 3.4 μg/ml) (Figure 3-4). Plasma CBG concentration on day 30 was almost 2-fold lower ($P < 0.01$) than that measured on days 10, 20, or 40. Pigs sampled during the prenatal period had higher plasma CBG concentrations as compared to those sampled during the postnatal period. In particular, plasma CBG level was very low in pigs on day 1 following birth.

Hepatic CBG mRNA expression. Hepatic CBG mRNA expression was highest ($P < 0.05$) on day 50 of gestation and decreased ($p < 0.01$) on day 90 of

Table 3-2. Body weight of pigs from birth to 40 days of age. Table values are least squares means ± SE.

Age (days)	1	3	10	20	30	40
Number of animal	8	8	8	8	8	8
Body weight (kg)	1.8 ± 0.3^{a}	2.0 ± 0.3^{a}	4.0 ± 0.3^{b}	7.1 ± 0.3^{c}	9.7 ± 0.3^{d}	13.7 ± 0.3^{e}

[a,b,c,d,e] Means within a row with uncommon superscripts differ ($P < 0.01$).

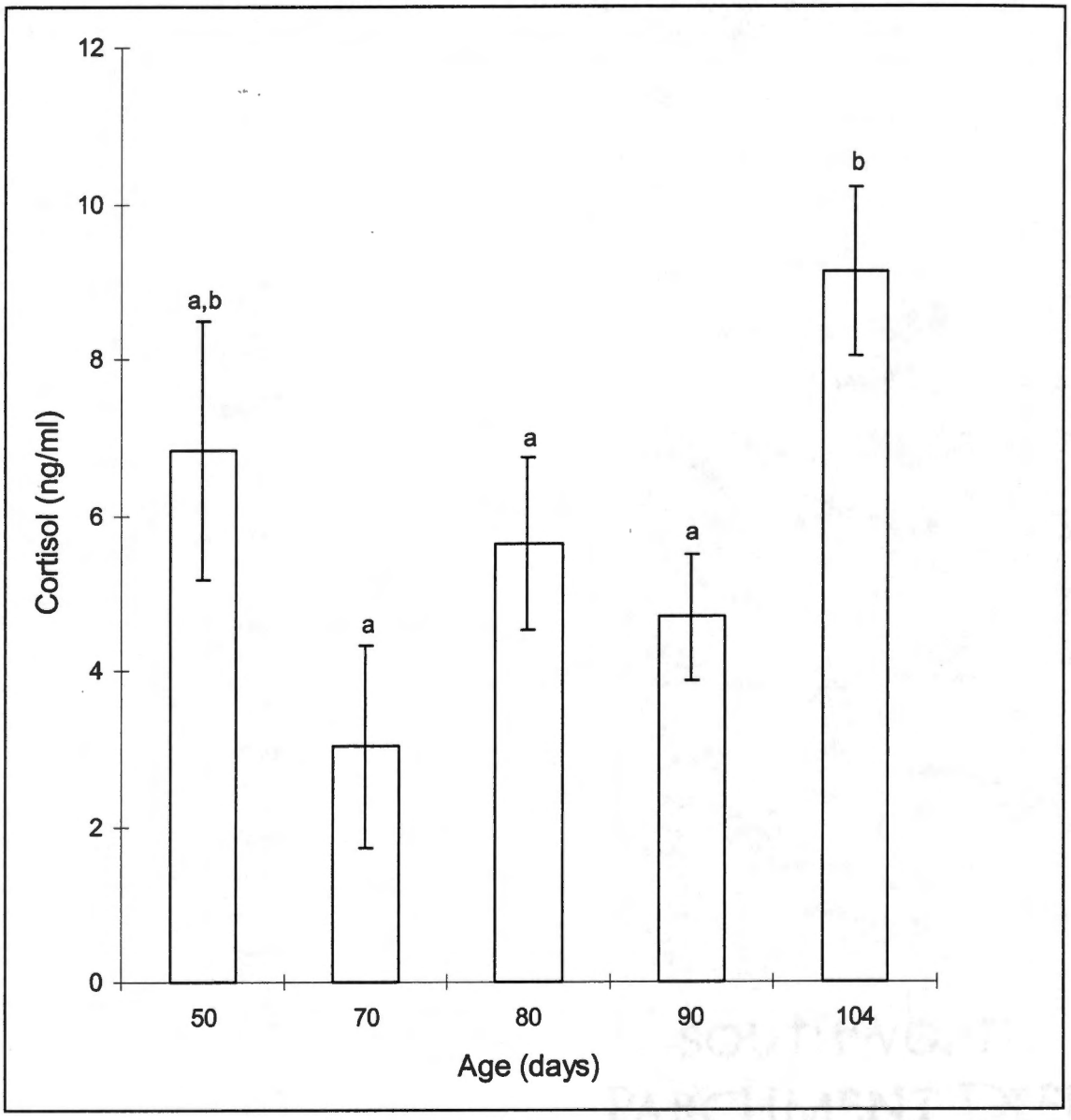


Figure 3-1. Plasma cortisol concentration in the fetal pig from 50 to 104 days of gestation. Cortisol concentration is expressed as nanogram per milliliter of plasma (ng/ml). Each column represents least squares means ± SE (n = 7-14). Least squares means with a different letter differ (P < 0.01).

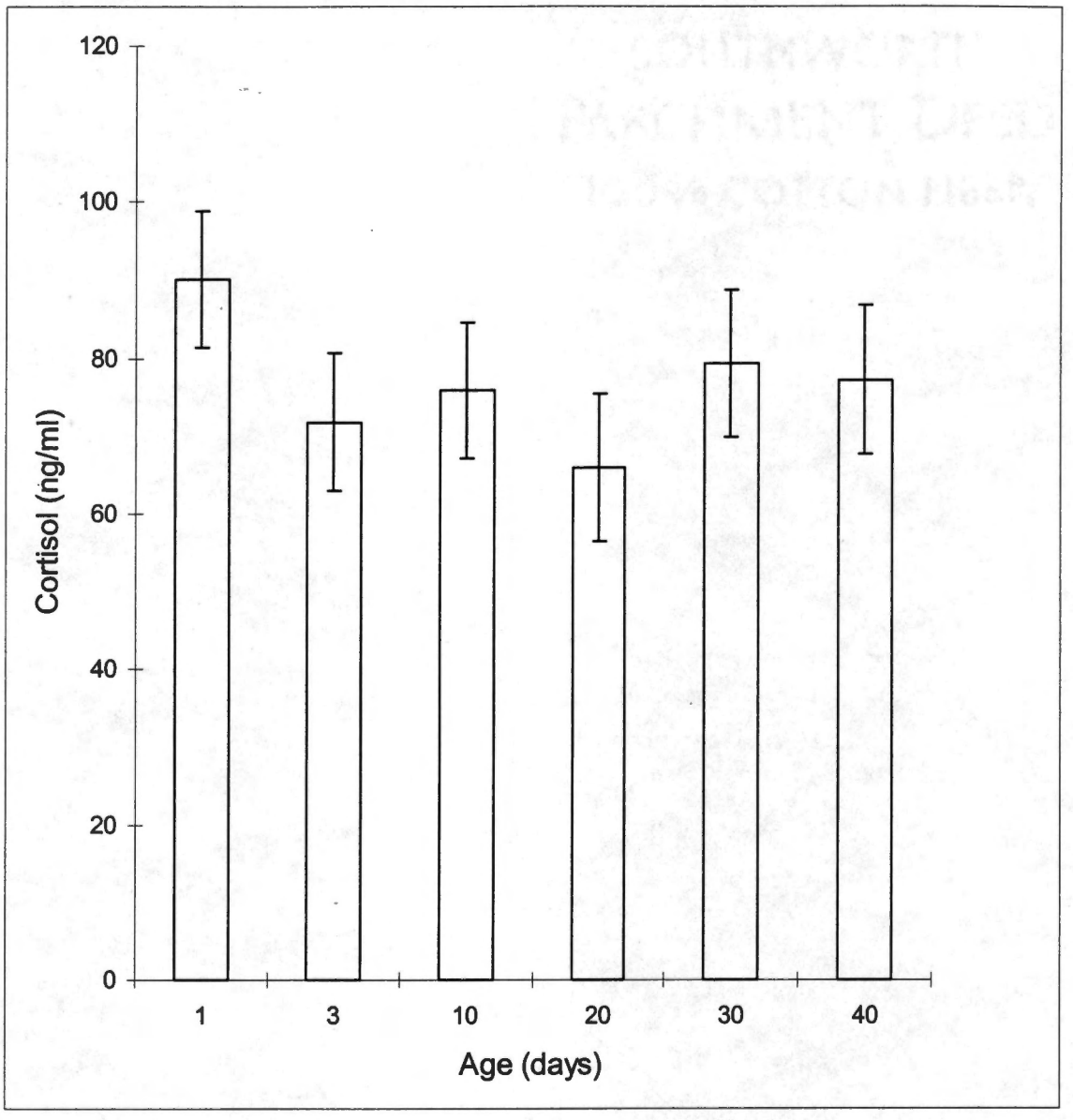


Figure 3-2. Plasma cortisol concentration in pigs from birth to 40 days of age. Cortisol concentration is expressed as nanogram per milliliter of plasma (ng/ml). Each column represents least squares means ± SE (n = 8).

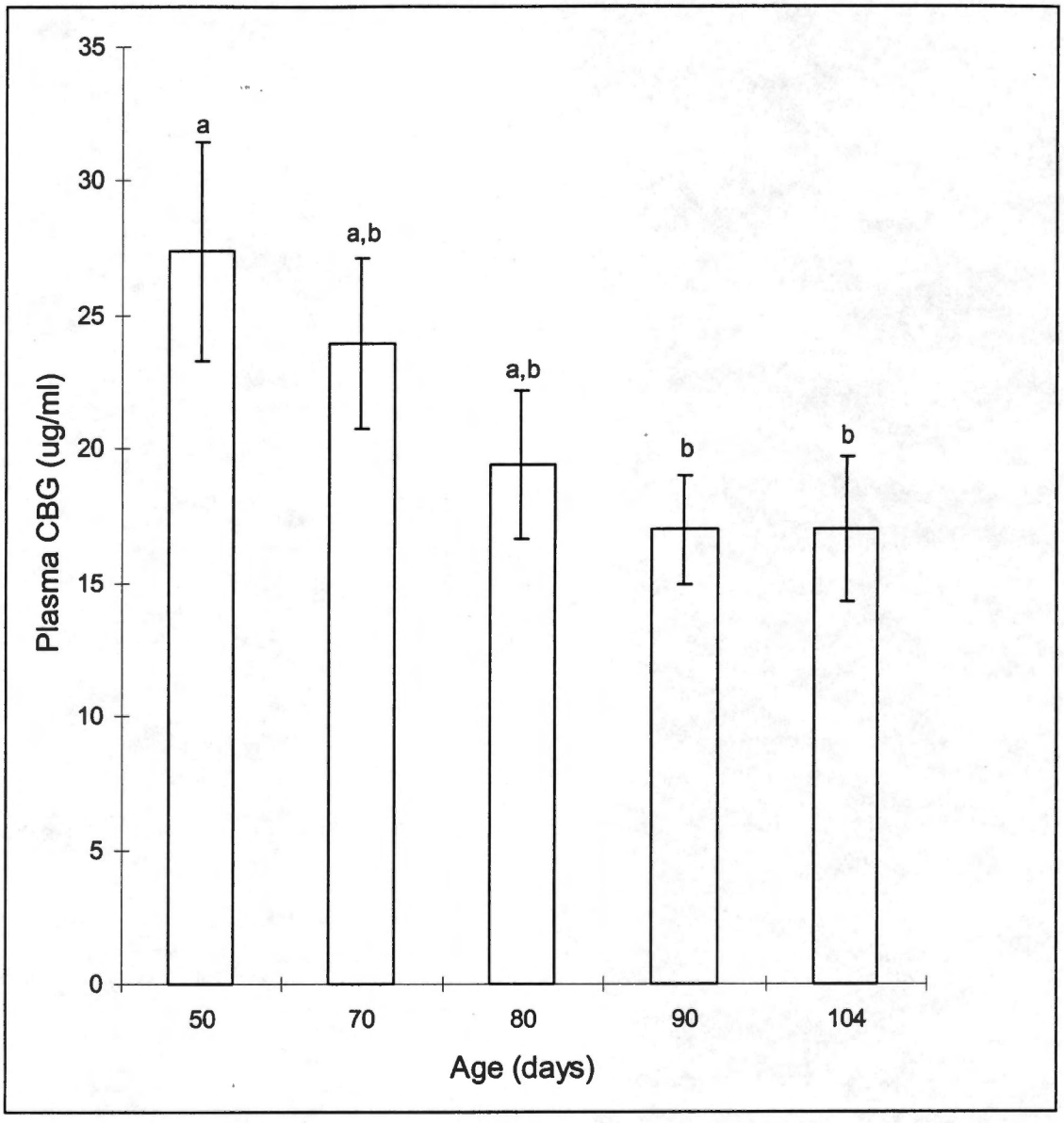


Figure 3-3. Plasma CBG concentration in the fetal pig from 50 to 104 days of gestation. CBG concentration is expressed as microgram per milliliter of plasma (μg/ml). Each column represents least squares means ± SE (n = 7-14). Least squares means with a different letter differ ($P < 0.01$).

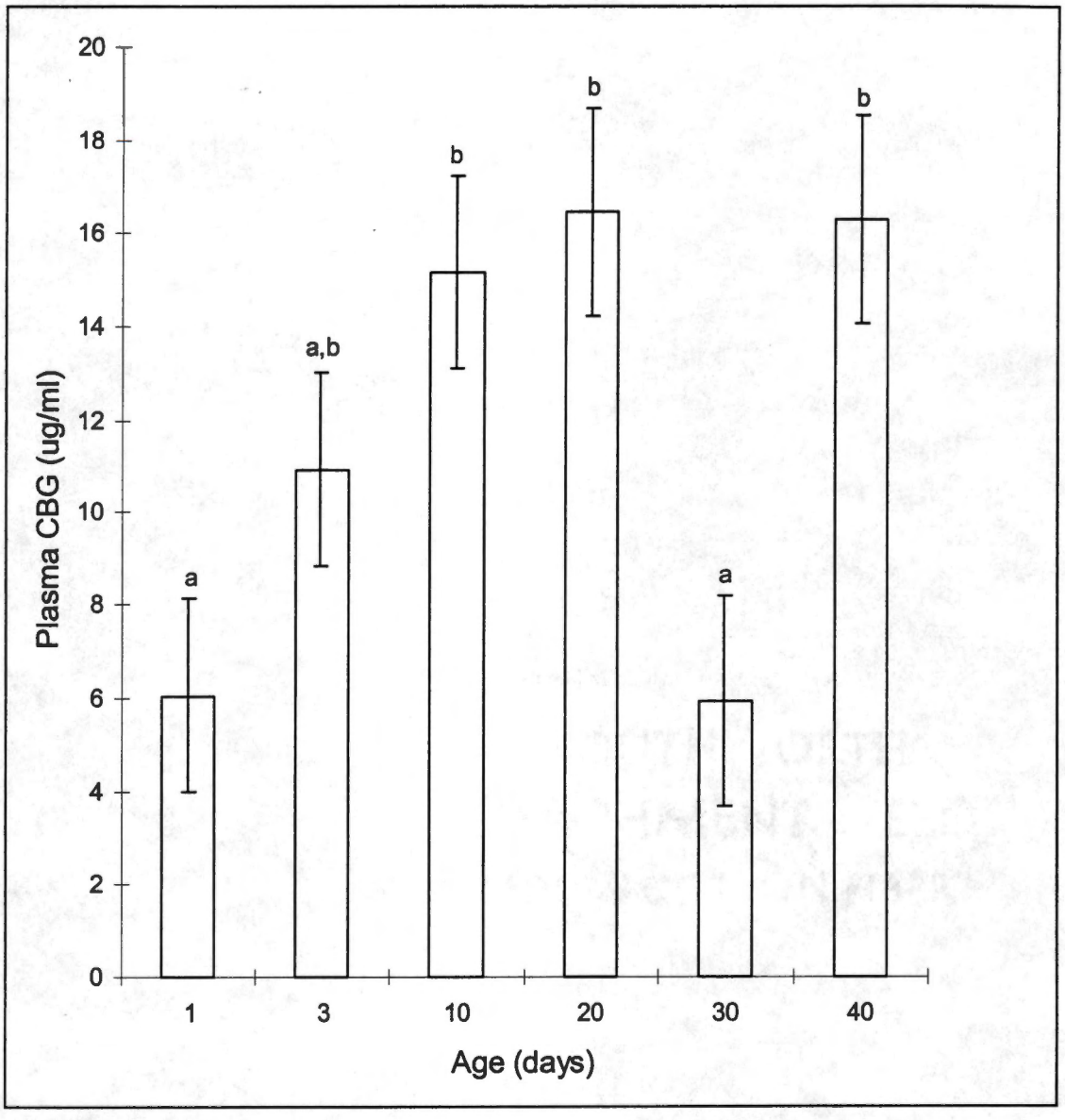


Figure 3-4. Plasma CBG concentration in pigs from birth to 40 days of age. CBG concentration is expressed as microgram per milliliter of plasma (μg/ml). Each column represents least squares means ± SE (n = 8). Least squares means with a different letter differ (P < 0.01).

gestation (Figure 3-5). There was no difference ($P > 0.1$) among pigs on days 70, 90 and 104 of gestation. Hepatic CBG mRNA expression was very low ($P < 0.01$) on day 3 and increased ($P < 0.01$) from day 10 to day 40 (Figure 3-6). However, pigs on d 30 did not show an increase ($P > 0.1$) in hepatic CBG mRNA expression compared to those on day 10 and day 20.

Relationships among plasma cortisol concentration, plasma CBG concentration, and hepatic CBG mRNA expression level. Correlation coefficients among plasma cortisol concentration, plasma CBG concentration, and hepatic CBG mRNA level of expression are summarized in Table 3-3. Plasma CBG concentration was negatively correlated with gestation age ($r = -0.51$, $P < 0.01$). Hepatic CBG mRNA expression was negatively correlated with gestation age ($r = -0.63$, $P < 0.01$). In postnatal pigs, hepatic CBG mRNA expression was correlated with postnatal age ($r = 0.75$, $P < 0.001$). Pigs having high plasma CBG concentration showed high expression levels of hepatic CBG mRNA during prenatal ($r = 0.34$, $P < 0.01$) and postnatal ($r = 0.40$, $P < 0.01$) sampling periods. Plasma cortisol concentration was not correlated ($P > 0.1$) with either plasma CBG concentration or hepatic CBG mRNA expression level during either period studied.

Discussion

The rates of fetal body weight gain increased with estimated gestational age. In the present study, the relationship ($r = 0.88$, $p < 0.01$) of estimated

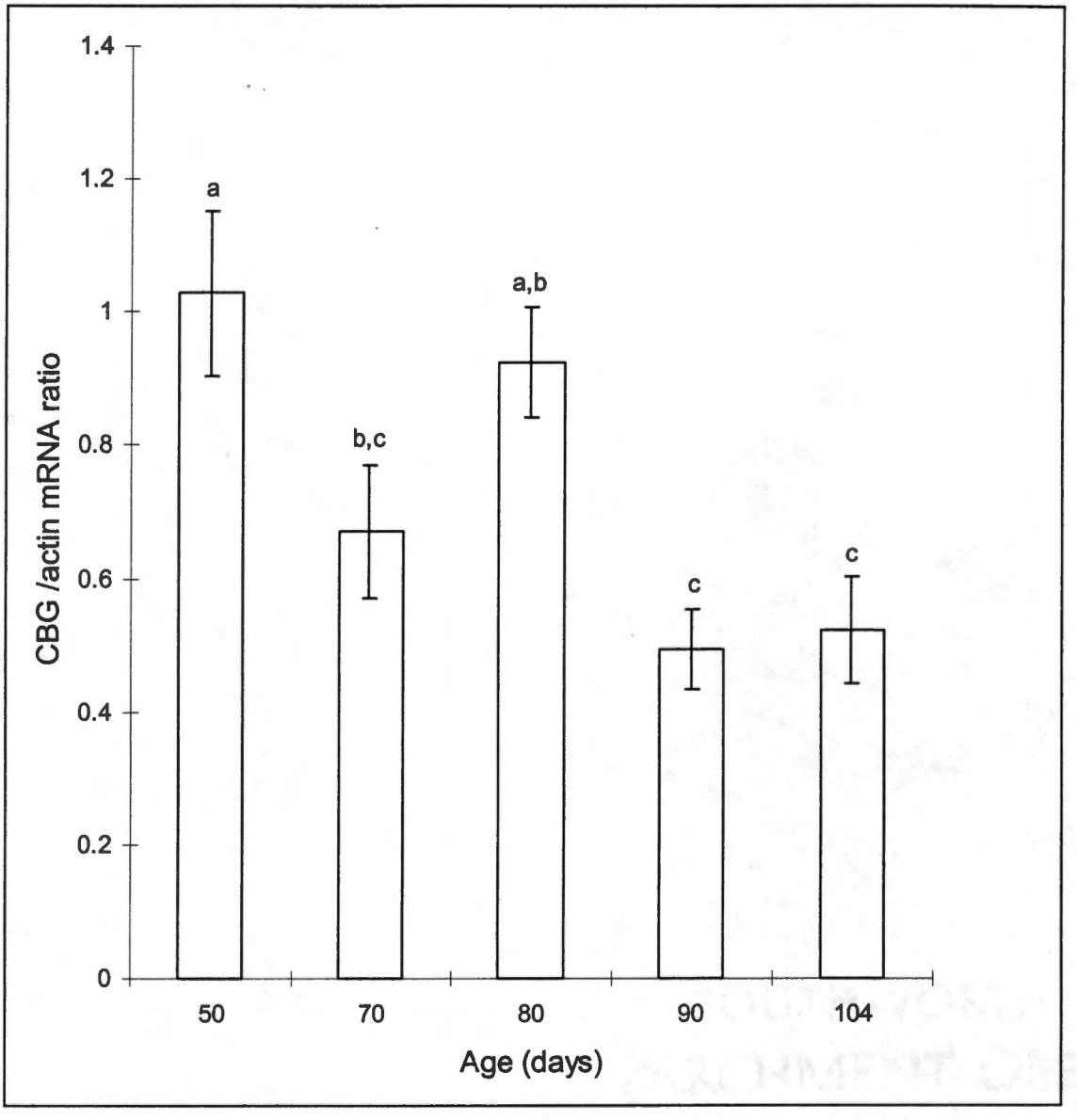


Figure 3-5. Hepatic CBG mRNA expression level in the fetal pig from 50 to 104 days of gestation. CBG mRNA expression level is expressed as the ratio of CBG mRNA level and actin mRNA levels (CBG/actin ratio). Each column represents least squares means ± SE (n = 7-14). Least squares means with a different letter differ ($P < 0.01$).

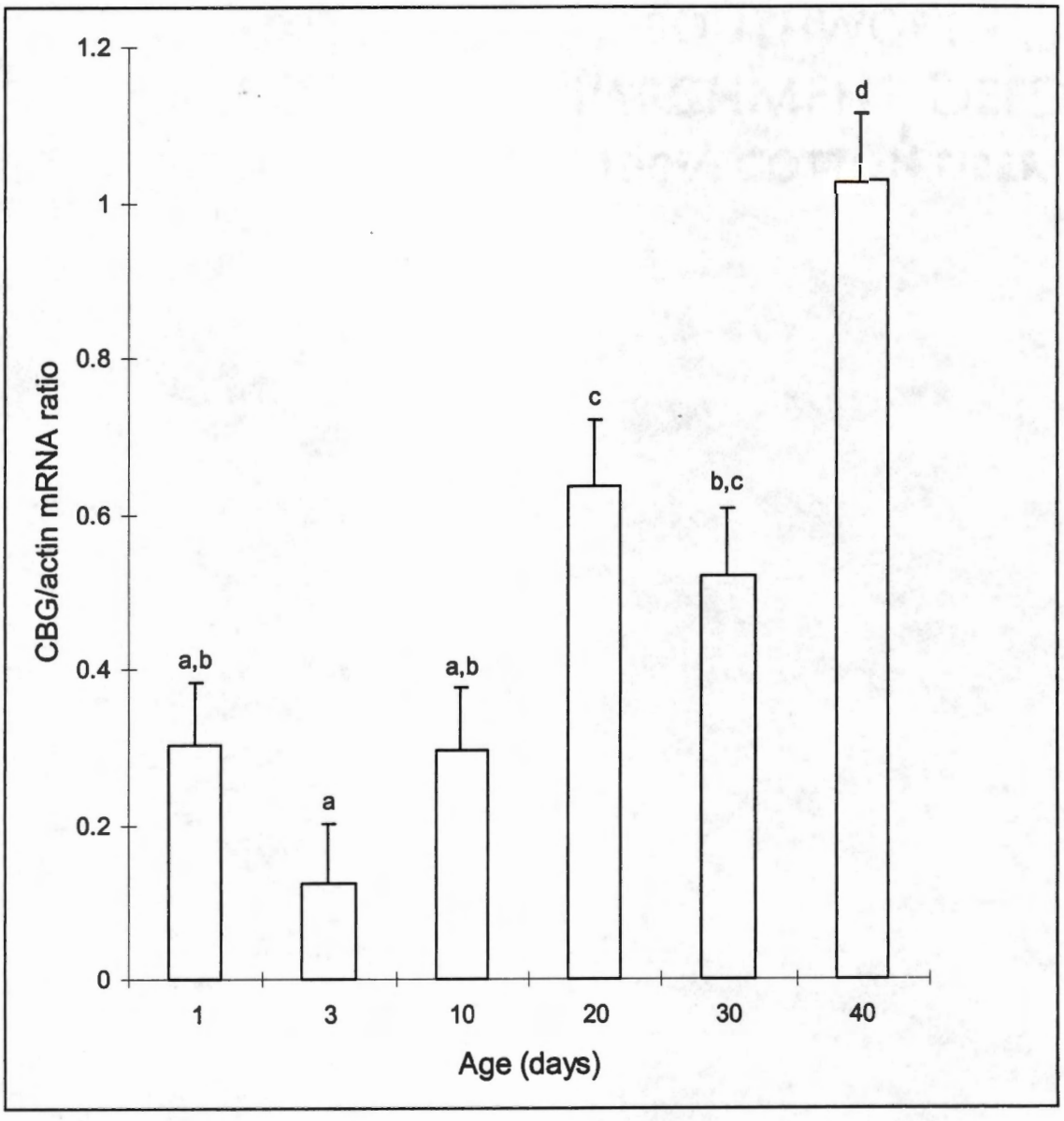


Figure 3-6. Hepatic CBG mRNA expression level in pigs from birth to 40 days of age. CBG mRNA expression level is expressed as the ratio of CBG mRNA level and actin mRNA levels (CBG/actin ratio). Each column represents least squares means ± SE (n = 8). Least squares means with a different letter differ ($P < 0.01$).

Table 3-3. Correlation coefficients between age, plasma cortisol, plasma CBG, and hepatic CBG mRNA levels in fetal (50 to 104 days of gestation) and postnatal (birth to 40 days of age) pigs.

	Fetal Pig				Postnatal Pig			
	Age	Cortisol	Plasma CBG	CBG mRNA	Age	Cortisol	Plasma CBG	CBG mRNA
Age	1.00	0.05	-0.51 **	-0.63 **	1.00	-0.06	0.22	0.75 **
Cortisol	0.05	1.00	-0.04	0.24	-0.06	1.00	-0.03	0.01
Plasma CBG	-0.51 **	-0.04	1.00	0.34 *	0.22	-0.03	1.00	0.40 **
CBG mRNA	-0.63 **	0.24	0.34 *	1.00	0.75 **	0.01	0.40 **	1.00

* $P < 0.05$, ** $P < 0.01$

gestation age with fetal body weight was similar to that reported previously (First and Bosc, 1979). A range of crown-rump lengths and body weights on the estimated gestation ages in this study is consistent with that reported in observations on fetal pigs of known ages (Marrable and Ashdown, 1967; Kattesh et al., 1997; Klemcke and Christenson, 1997). Body weights of pigs during the postnatal period were similar to that reported previously (Kattesh et al., 1990).

In the present study, however, the rate of body weight gain in postnatal pigs between day 20 and day 30 were less than that between day 10 and day 40. Stanton and Mueller (1976) reported that weaning of swine at day 21 of age is a stressful experience and decreases the rate of body weight gain. Therefore, the reduced rate of body weight gain between day 20 and day 30 of age is possibly the result of weaning that occurred on day 23 of age.

Plasma cortisol concentration in fetal pigs was low between day 70 and day 90 of gestation, and increased on day 104 of gestation. An increase in fetal cortisol concentration in late gestation is associated with the maturation of fetal organs (Liggins, 1994), growth of the fetus (Klemcke and Christenson, 1997), and the induction of parturition in swine (First and Bose, 1979). The present study showed an increase in plasma cortisol on day 104 of gestation whereas other studies showed a rise in fetal cortisol on day 108 (Randall, 1983), day 100 (Klemcke and Christenson, 1997) or day 110 (Kattesh et al., 1997) of gestation. The increase in plasma cortisol concentration in fetal pigs late in gestation may result from an increase in hyperplasia of the zona fasiculata rather than an increase in sensitivity of the fetal adrenal to ACTH (Lohse and First, 1981).

Onset and magnitude of the initial increase in plasma cortisol has been reported to vary both between individuals within a litter and between litters (Randall, 1983). Although there was a high variation between individuals in the present study, plasma cortisol concentration in fetal pigs decreased two-fold between day 50 and day 70 of gestation. Klemcke and Christenson (1997) similarly showed that fetal plasma cortisol concentration decreased in pigs by 30% between days 50 and 75 of gestation even though ACTH concentration increased. The authors suggested that this decrease was due in part to a reduction in adrenal cortisol secretion and the ability of the porcine fetal adrenal to respond to ACTH.

The concentration of plasma cortisol in the pig during the postnatal period remained relatively high and unchanged in contrast to that reported earlier (Kattesh et al., 1990). Adrenocortical activity in pigs is highest at birth and decreases with age following birth (Dvorak, 1972; Kattesh et al., 1990). McCauley and Hartmann (1984) found a high concentration of cortisol in the piglet at birth followed by a rapid decline to adult values within 5 days after birth, suggesting a profound change in cortisol metabolism at this time. In the present study, blood samples were collected while pigs were maintained under general anesthesia. The anesthesia and surgery itself is known to cause circulating cortisol levels to be elevated (Dalin et al., 1993). Also, the absolute plasma cortisol values for the postnatal pigs used in the present study were quite high compared to thoser reported previously in unanesthetized pigs (Kattesh et al., 1990). Hence, the elevation in cortisol seen here might be due to anesthesia, and may explain our failure to detect a decline in cortisol levels by day 3 following

birth as reported previously (McCauley and Hartmann, 1984; Kattesh et al., 1990).

The present study is the first to document changes in plasma CBG concentration in the fetal pig. CBG levels were relatively high during mid-gestation and then declined through day 104 of gestation. In the fetal rat, plasma CBG levels fall from 19 to 21 days of gestation (Smith and Hammond, 1991). In the fetal rabbit, serum CBG levels also show a progressive decline beginning 9 days prepartum (Seralini et al., 1990). In contrast, plasma CBG binding capacity (CBC) that has been used to measure plasma CBG level (Ballard, 1979) increased during the final 10 to 15 days of pregnancy in fetal sheep (Jacobs et al., 1991) with a concomitant rise in cortisol (Ballard et al., 1983). It is theorized that the glucocorticoid-induced increase in plasma CBG levels in fetal sheep is associated with increased half-life due to alterations in glycosylation as well as increased hepatic biosynthesis (Berdusco et al., 1993). Kattesh and coworkers found that plasma CBG binding capacity (CBC) measured in the fetal pig was higher on day 113 compared to day 105 of gestation. Therefore, the increase in plasma CBG level in fetal pigs near term (Kattesh et al., 1997) may be related in part to a rise in plasma cortisol level on day 104 in the present study.

Plasma CBG concentration increased from day 1 to day 10 following birth and then remained constant until day 40 with the exception of a significant drop on day 30. In the rat, plasma CBG levels are very low during the first 9 days following birth and significantly increase between day 9 and day 12 until a plateau is reached on day 24 (Henning, 1978). Kattesh and Roberts (1993)

observed in unweaned pigs that plasma CBG concentration decreased from day 3 to day 7 and significantly increased between day 21 and day 28 of age. Weaning of swine at day 21 of age is a stressful experience (Stanton and Mueller, 1976). In horses, stress resulting from social instability decreased plasma CBG level (Alexander and Irvine, 1998). The decline in plasma CBG concentration on day 30 in the present study may be the result of stress associated with weaning the pigs on day 23.

During gestation, hepatic CBG mRNA expression in the fetal pig decreased with age. This observation is consistent with that in the fetal rabbit (Seralini et al., 1990) and rat (Smith and Hammond, 1991). Seralini et al. (1990) suggested that a progressive decline in fetal rabbit hepatic CBG mRNA from day 22 of pregnancy until delivery may be explained by a developmental variation in the production of transcription factors that compete with the glucocorticoid receptor as a ligand for DNA-binding sites in the CBG gene promoter. Although glucocorticoids suppress hepatic CBG mRNA levels in adult rats (Smith and Hammond, 1992), the fetal CBG gene is unresponsive to glucocorticoids before day 19 of gestation (Smith and Hammond, 1991) because fetal plasma CBG levels was not affected by dexamethasone treatment at this time (Van Baelen et al., 1977). Injection of the synthetic glucocorticoid dexamethasone increased hepatic CBG mRNA levels in fetal sheep on day 130 of gestation but decreased it in adult sheep (Berdusco et al., 1993). Therefore, the CBG gene expression may be regulated differently during development. Although the physiological significance of the decline in fetal CBG expression is unknown, the resulting

decrease in plasma CBG levels may contribute to an increase in free glucocorticoid levels in the fetus (Smith and Hammond, 1991), and may influence maturational events of most organ systems (Liggins, 1994).

The ontogeny of hepatic CBG mRNA expression in postnatal pigs follows a pattern similar to that reported in the rat (Smith and Hammond, 1991) and mouse (Scrocchi et al., 1993). In male rat, dexamethasone treatment reduced the rate of CBG gene transcription, and thyroxine treatment increased CBG mRNA stability rather than the rate of CBG gene transcription (Smith and Hammond, 1992). In the sheep, glucocorticoids suppress hepatic CBG mRNA expression (Berdusco et al., 1993). In the present study, plasma cortisol level was not changed during the postnatal period and plasma CBG concentration increased from day 1 to day 10 following birth, which may result in a decrease in the fraction of free cortisol in plasma. Kattesh et al. (1990) also found that percent unbound cortisol decreased from day 3 to day 40 following birth. Therefore, an increase in hepatic CBG mRNA expression as was observed in this study may be due to the reduced inhibitory effect of the lowered free cortisol on hepatic CBG mRNA expression. Likewise, an increase in cortisol associated with the stress of weaning may have resulted in the suppression in hepatic CBG mRNA expression seen on day 30 following birth.

The present study is the first to document the relationship between plasma CBG concentration and hepatic CBG gene expression during prenatal and postnatal development in the pig. In the fetal pig, plasma CBG concentration and hepatic CBG mRNA expression decreased with gestational age similar to that

reported in rabbits (Seralini et al., 1990) and rats (Smith and Hammond, 1991). In the present study, furthermore, plasma CBG concentration in fetal pig was positively correlated to hepatic CBG mRNA expression. Therefore, it is likely that plasma CBG concentration in the fetal pig may be determined by the hepatic CBG mRNA expression. In the present study, hepatic CBG mRNA expression increased with postnatal age, but plasma CBG was unrelated to postnatal age. In the rat, adult CBG mRNA levels were attained by 3 weeks of age, but serum CBG concentrations did not reach adult values for an additional 3 weeks, which may be due to age-dependent alterations in the clearance of CBG (Smith and Hammond 1991). The present study suggests that plasma CBG concentration in the postnatal pig may be determined by other factors such as metabolic clearance and/or transfer to the extravascular space.

Understanding the relationships between plasma CBG concentration and hepatic CBG biosynthesis may be helpful in determining the mechanism regulating plasma CBG concentrations as well as the bioavailability of glucocorticoid during prenatal and postnatal development. The present study suggests that plasma CBG concentration in the pig may be regulated differently according to the animal's stages of development.

CHAPTER 4. HEPATIC CORTICOSTEROID-BINDING GLOBULIN (CBG) mRNA EXPRESSION AND PLASMA CBG LEVELS IN PIGS IN RESPONSE TO SOCIAL AND HEAT STRESS

Abstract

Plasma cortisol, CBG, hepatic CBG expression, and other physiological and behavioral measures of stress were studied in pigs in response to elevated temperature in conjunction with establishing social hierarchy. Twenty-four pigs (four pigs/litter) were weaned at 25 days of age and housed by litter for 2 weeks at 23 ± 2°C. On day 0, animals were weighed and placed under general anesthesia for collection of blood (10 ml) and liver tissue (~100 mg). On day 1, three pigs of similar weight (23 ± 0.9 kg) but from different litters were allotted to eight nursery pens within two environmentally controlled rooms (12 animals/room). From days 1 to 7 (treatment period), one room was maintained at 23 ± 2°C (control, CON) and the other at 33 ± 2°C (heat treatment, HEAT). From days 8 to 14 (recovery period), both rooms were maintained at 23 ± 2°C. Animals were videotaped for 72 hours beginning on days 1 and 8 to document behavioral changes in response to room temperature and to determine social order. Blood and liver tissue were collected again on days 7 and 14. Plasma haptoglobin increased (467 ± 123 vs 763 ± 113 μg/ml; day 0 vs day 7, $P < 0.05$)

while cortisol and CBG decreased (99.3 ± 8.3 vs 85.1 ± 8.3 ng/ml, 11.4 ± 1.1 vs 9.9 ± 1.1 μg/ml; day 0 vs day 7, respectively, $P < 0.05$) in the HEAT pigs. Hepatic CBG mRNA level and neutrophil:lymphocyte ratio were not affected ($P > 0.1$) by treatment. HEAT pigs displayed increased ($P < 0.01$) drinking but reduced feeding ($P < 0.01$) and lying in contact with other pigs ($P < 0.05$) behaviors. Average daily gain of body weight (ADG) tended ($P = 0.06$) to be lower for HEAT (0.64 ± 0.06 kg/d) compared to CON (0.82 ± 0.06 kg/d) pigs. During recovery period, HEAT pigs had similar ($P > 0.1$) ADG, plasma cortisol, CBG, haptoglobin, and drinking and feeding but increased ($P < 0.01$) lying with contact behaviors compared to CON pigs. Measured physiological and behavioral responses were not related to social status. These results indicate that reduced circulating levels of cortisol and CBG in pigs following 7-day exposure to elevated temperature may not be determined by hepatic CBG mRNA expression.

Introduction

Stress has been defined as a biological response elicited when an individual perceives a threat to its homeostasis (Moberg, 2000). Swine are exposed to various stressful conditions including heat and humidity (Marple et al., 1972; Morrow-Tesch et al., 1994), feed and water deprivation (Parrott and Mission, 1989), social pressure (Mormede et al., 1990; Hicks et al., 1998), and mixing (Dalin et al., 1993; Ekkel et al., 1997). The stress response exhibits itself

in both significant behavioral changes and physiological changes (Nagaraja and Jeganathan, 1999). A behavioral response is the first and undoubtedly most biologically economical response (Moberg, 2000). Pigs respond with different behaviors to different stressors (Hicks, 1998). One of the most apparent physiological responses observed following stress is the change in plasma cortisol levels through activation of the hypothalamic-pituitary-adrenal axis (Ottenweller et al., 1992).

Mixing pigs induces high levels of aggression among the animals as they attempt to establish a new social rank (Ekkel et al., 1997), which leads to a chronic social stress. Social stress has significant affects on plasma cortisol, globulin, acute-phase proteins, and body weight (Hicks et al., 1998). Pigs exposed to elevated temperatures and humidity exhibit changes in behavior, food intake, body weight, endocrine, and immune status (Marple et al., 1972; Kelley, 1980; Hicks et al., 1998; Matteri et al., 2000). Heat and social stress may have interaction in their effects on pigs (McGlone et al., 1987; Morrow-Tesch et al., 1994). Furthermore, behavioral and hormonal responses to stressors do not occur independently but have mutual relationships among each other (Dantzer and Mormede, 1983).

Corticosteroid-binding globulin (CBG) is a circulating glycoprotein produced mainly by the liver that regulates the bioavailability of cortisol (Rosner, 1990) and plasma cortisol levels (Schroeder and Henning, 1989). Plasma CBG level is determined by its biosynthesis, degradation and/or transfer to extravascular spaces, and may be influenced by age, physiological conditions, or

stressful situations (Heyns and Coolens, 1988). Since CBG has only one steroid-binding site per molecule (Mickelson et al., 1982) and CBG binding sites become saturated at higher levels of steroid, the binding capacity of CBG in plasma has been used to measure the plasma CBG level (Ballard, 1979). In rats, plasma CBG binding capacity was significantly reduced following acute stress (Tannenbaum et al., 1997). This decrease in CBG binding capacity within the circulation was associated with an increase in free corticosterone levels (Tannenbaum et al., 1997). Earlier, Bassett (1986) reported finding short-term increases in plasma corticosteroid-binding activity in rats following brief exposure to acute stress. Therefore, it is still unclear how plasma CBG level is regulated during stressful situations and how the change in plasma CBG level affects plasma cortisol levels.

The objective of this study was to examine the relationships among plasma cortisol and CBG levels, and hepatic CBG mRNA expression in pigs subjected to elevated environmental temperature in conjunction with establishing social hierarchy.

Materials and methods

Materials. PGI EZ one handed biopsy needles (14-gauge) were purchased from Product Group International, Inc. (Lyons CO). Hema-Stat 3 stain set was purchased from Biochemical Sciences, Inc. (Swedesboro, NJ). Coat-A-Count RIA kit was purchased from Diagnostic Products Corp. (Los Angeles, CA).

ELISA plates were purchased from Corning Costar Corp. (Oneonta, NY). p-Nitrophenyl-phosphate was purchased from Kirkegard & Perry Lab (Gaithersburg, MD). Porcine Haptoglobin Measurement Kit was purchased from Cardiotech services, Inc. (Louisville, KY). Tri-Reagent, yeast total RNA, Sephadex G-50, and anti-rabbit IgG-alkaline phosphatase conjugate were purchased from Sigma Chemical (St Louis, MO). Nylon transfer membrane was purchased from Schicher & Schuell (Keene, NH). The radioisotope [α-^{32}P] dCTP was purchased from Du Pont NEN (Boston, MA). A random primer labeling kit was purchased from Stratagene (La Jolla, CA). Human β-actin cDNA control probe was purchased from Clontech (Palo Alto, CA). Koda X-Omat film was purchased from Eastman Kodak (Rochester, NY). All other chemicals were purchased from Sigma (St Louis, MO) and Fisher Scientific (Pittsburgh, PA).

Animals. Twenty-four crossbred pigs (Landrace, Duroc and Hampshire) were weaned at 25 days of age from six sows (3 or 6 pigs per sow). Pigs were weighed individually on day 43 of age and transported from the UT swine unit to the Johnson Animal Research and Teaching Unit (JARTU). Pigs were kept in littermate groups and acclimated to environmentally controlled rooms (23 $\pm$ 2°C) for 2 weeks until they were assigned to treatment. All pigs were given ad libitum access to a commercial diet (CO-OP Zipper Feed 25-50 with ASP; 18% crude protein, 1.15% lysine) and given free access to water. Pigs were maintained on a constant 24 hours photoperiod throughout the entire experimental period. Length of photoperiod has no effect on growth or development of pigs at this age

(McGlone et al., 1988). Animal use in this study was approved by the University of Tennessee Animal Care and Use Committee.

Experimental design. On day 0, all animals were weighed and the first blood and liver tissue samples were collected as described below. On day 1, three unacquainted pigs of similar weight (23 ± 0.9 kg) from different litters were allotted to each of eight nursery pens within two environmentally controlled rooms (12 animals per room). From days 1 to 7 (treatment period), one room was maintained at 23 ± 2°C (CON) and the other was maintained at 33 ± 2°C (HEAT). From days 8 to 14 (recovery period), both rooms were maintained at 23 ± 2°C. Body weight of each pig was recorded, and additional blood and liver tissue samples were collected from each pig on days 7 and 14.

Behavioral Measures. All pigs were video-taped for 72 hours beginning on days 1 and 8 to determine social hierarchy and to document behavioral changes during the first three days of treatment and recovery. Pigs were individually marked with farm animal crayon markers (All Weather Painstik, La-Co Industries, Inc., Elk Grove Village, IL). Video cameras (WV CP412, Panasonic Matsushita Electric Corp., Secaucus, NJ) hung from the ceiling allowed for filming of the four pens with one camera (Figure 3-1). Behaviors were recorded continuously for 72 hours (2.6 frame per sec) using a time lapse recorder (AG-6740P, Panasonic Matsushita Electric Corp., Secaucus, NJ).

For the determination of social hierarchy, tapes were viewed at real-time speed using a time lapse recorder. Length of fight, pigs involved and winner were recorded for each observed fight. The dominance hierarchy was divided into three ranks: dominant, intermediate, and subordinate (McGlone, 1985). When pigs were observed fighting, the video tape was slowed to record duration of each fight. Pigs that pursued a second pig or caused the second pig to turn away from it were determined as the winner. Animals were assigned to a rank using a dominance matrix where the numbers of fights between each pair of pigs in a pen were listed along with the winner (Martin and Bateson, 1986; McGlone, 1985). Since each pen had three animals, only one animal was designated in each social rank.

Each animal was viewed in one frame of videotape every 15 minutes and the frequency of the following behaviors was observed:

Standing/walking - the pig was up on all four feet, either standing or walking.

Drinking - the pig's mouth/snout was touching the waterer.

Feeding - the pig's head was in the feeder.

Lying with contact - the pig was lying down or sitting in physical contact with another animal.

Lying without contact - the pig was lying down or sitting without physical contact with another animal.

Plasma and liver tissue collection. On days 0, 7 and 14, each pig was removed from its pen, weighed, and pre-anesthetized by intramuscular injection

of a solution of 30 parts Ketamine (100 mg/ml) and one part Acepromazine (10 mg/ml) at a dose of 0.2 ml/kg body weight. The animal was moved to the large animal surgery room and placed under general anesthesia by closed circuit halothane administration using a gas inhalation machine.

A blood sample (10 ml) was collected from the anesthetized pig via puncture of the external jugular vein and the sample placed in a vacutainer tube containing 2% solution of the anticoagulant ethylenediaminetetraacetate (EDTA). Blood smears were made using whole blood for differential white cell counts. Following centrifugation at 2,000 x g for 10 minutes, plasma was recovered and stored at -20°C until analyzed for cortisol, haptoglobin, and CBG concentrations.

Percutaneous ultrasound-guided liver biopsies were performed on each pig while under general anesthesia (Martino et al., 1984; Plecha et al., 1997). PGI EZ one handed biopsy needles were used for all biopsies. Briefly, the animal was placed in dorsal recumbency, and a 10 cm square area caudal to the xiphoid cartilage was shaved, cleaned with 70% isopropanol, and examined with an Aloka 900 ultrasound (Corometrics Medical Systems, Wallingford, CT) equipped with a 7.5 MHz convex transducer. The insertion point of the biopsy needle was selected following ultrasound observation of the vascularity of the liver. A small incision was made, and the biopsy needle was guided into the liver under visualization using ultrasound. Two or three biopsies, providing 30 to 50 mg of tissue/ biopsy, were performed on each animal. The samples were quickly homogenized in 3 ml of Tri Reagent solution. The animals were allowed to recover and then returned to their original pens.

Differential white cell counts. Whole blood slides were stained using Hema-Stat 3 stain set and observed under a light microscope at 600 x magnification. A total of 100 white blood cells were counted and identified as lymphocytes, neutrophils, monocytes, eosinophils or basophils (Diggs et al., 1978). The neutrophil to lymphocyte ratio (N:L) proposed as an indicator of a stress (Widowski et al., 1989) was calculated for each sample.

Plasma cortisol analysis. Cortisol concentrations were determined using the radioimmunoassay (RIA) procedure of Coat-A-Count as described previously (Chapter 3; Appendix C). Cortisol concentration was expressed as nanogram per milliliter (ng/ml). Inter- and intra-assay coefficient of variation was 9.1 % and 3.6 %, respectively.

Plasma CBG analysis. The amount of CBG in plasma was measured by a direct enzyme-linked immunosorbent assay (ELISA) for porcine CBG (pCBG) developed by Kattesh and Roberts (1993) as described previously (Chapter 3, Appendix D). Plasma CBG concentration was expressed as microgram per milliliter (μg/ml). Inter- and intra-assay coefficient of variation was 13.4 % and 11.7 %, respectively.

Plasma haptoglobin analysis. Plasma concentration of haptoglobin was determined by a single radial immunodiffusion (SRID) using a commercially

available kit (Cardiotech Services, Louisville, KY). The diameter of the precipitation ring was measured after incubation at 32°C for 24 hours. Plasma samples or standards were placed in gelatin wells containing porcine anti-haptoglobin antibodies. The diameter of the ring was inversely related to the amount of plasma haptoglobin. Results were evaluated using a logarithmic regression equation ($Y = -695.4 + 167.8$, $r^2 = 0.98$) to determine plasma concentration of haptoglobin in each sample using known standards. Intra-assay coefficient of variation of duplicate estimates was 4.8 %.

RNA extraction. Total RNA was extracted from liver tissue samples with Tri-Reagent as described previously (Chapter 2, Appendix A).

Slot Blot Analysis. The expression of CBG mRNA was determined by Slot blot procedure modified from that of Sambrook et al.(1989; see Appendix E). A partial porcine CBG cDNA developed previously (Chapter 2) was used as a probe to detect CBG mRNA in pig liver tissue. CBG mRNA level was expressed relative to β-actin mRNA level.

Statistics. The study was analyzed as a completely random, repeated measures design using PROC MIXED procedure of SAS (SAS, 2000) with the following independent variables (main plots): treatment, social status and treatment x social status interaction. Social status was nested within treatment pens. Variance among pens was used as the error term to test main plot effects.

Additional (sub-plot) factors of period and the interaction of period with each main-effect treatment or social status were included in the analysis. Residual error was used to test all sub-plot effects. Behavioral data were transformed to log form to normalize the data for statistical analysis. Data were represented as least squares means with standard errors. Least squares means were separated using Fishers Least Significant Difference test (Norman and Streiner, 1994). Pearson correlation coefficients (Norman and Streiner, 1994) were calculated to describe relationships among the dependent variables.

Results

Body weights and average daily gain of pigs. Body weight of pigs after a 7-day treatment period and after a 7-day recovery period are summarized in Table 4-1. There were no differences ($P > 0.1$) in body weights between CON and HEAT pigs following either period. ADG for HEAT pigs increased ($P < 0.05$) from 0.64 ± 0.06 kg/d as measured over days 1 to 7, to 0.84 ± 0.06 over days 8 to 14. ADG did not differ among CON pigs between the treatment and the recovery period ($P > 0.1$).

The effect of social rank on body weight is summarized in Table 4-2. Subordinate pigs (30.4 ± 1.1 kg) were heavier ($P < 0.05$) than dominant pigs (28.0 ± 1.1 kg) after the 7-day treatment period. There was no significant difference in body weights among dominant, intermediate, and subordinate pigs

Table 4-1. Body weights and average daily gain of pigs before treatment (Before; CON and HEAT at 23 ± 2°C on day 0), after a 7-day treatment (Treatment; CON at 23 ± 2°C vs HEAT at 33 ± 2°C from day 1 to day 7) and after a 7-day recovery period (Recovery; CON and HEAT at 23 ± 2°C from day 8 to day 14). Table values are least squares means ± SE.

Measures	Before		Treatment		Recovery	
	CON	HEAT	CON	HEAT	CON	HEAT
Number of animasl	12	12	12	12	12	12
Body weight (kg)	23.9 ± 1.3^{a}	24.7 ± 1.3^{a}	29.6 ± 1.3^{b}	29.2 ± 1.3^{b}	34.7 ± 1.3^{c}	35.1 ± 1.3^{c}
Average daily gain (kg/day)	+++	+++	0.8 ± 0.1^{ab}	0.6 ± 0.1^{a}	0.7 ± 0.1^{ab}	0.8 ± 0.1^{b}

[a,b,c] Means within a row with uncommon superscripts differ ($P < 0.05$).

+++ Data for average daily gain of pigs before treatment were not available.

Table 4-2. Body weights and average daily gains of dominant (Dom.), intermediate (Int.), and subordinate (Sub.) pigs before treatment (Before; CON and HEAT at 23 ± 2°C on day 0), after a 7-day treatment (Treatment; CON at 23 ± 2°C vs HEAT at 33 ± 2°C from day 1 to day 7) and after a 7-day recovery period (Recovery; CON and HEAT at 23 ± 2°C from day 8 to day 14). Table values are least squares means ± SE.

Mearures	Before			Treatment			Recovery		
	Dom.	Int.	Sub.	Dom.	Int.	Sub.	Dom.	Int.	Sub.
Number of animals	8	8	8	8	8	8	8	8	8
Body weight (kg)	23.7 ± 1.1^{a}	24.2 ± 1.1^{a}	25.0 ± 1.1^{a}	28.0 ± 1.1^{b}	29.9 ± 1.1^{bc}	30.4 ± 1.1^{c}	33.6 ± 1.1^{d}	35.3 ± 1.1^{d}	35.7 ± 1.1^{d}
Average daily gain (kg/day)	+++	+++	+++	0.61 ± 0.06^{a}	0.81 ± 0.06^{b}	0.77 ± 0.06^{ab}	0.81 ± 0.06^{ab}	0.78 ± 0.06^{ab}	0.75 ± 0.06^{ab}

[a,b,c,d] Means within a row with uncommon superscripts differ ($P < 0.05$).

+++ Data for average daily gain of pigs before treatment were not available.

following the recovery period. ADG for intermediate pigs was greater ($P < 0.05$) than that of dominant pigs over the treatment period. However, there was no significant difference in average daily gain among dominant, intermediate, and subordinate pigs over the recovery period.

Behaviors of pigs. Frequencies of behaviors of pigs observed from days 1 - 3 or from days 8 - 10 are summarized in Table 4-3. HEAT pigs displayed increased ($P < 0.05$) frequency of feeding and lying with contact with other pigs during the treatment period as compared to the recovery period. The frequency of drinking, standing/walking, and lying without contact with other pigs decreased ($P < 0.05$) in HEAT pigs during the recovery period compared to that observed during the treatment period. CON pigs showed increased ($P < 0.05$) frequency of drinking behaviors during the recovery period as compared to the treatment period, but did not show any differences in other behaviors between the treatment and recovery periods. During the treatment period, HEAT pigs displayed a greater ($P < 0.05$) frequency of drinking and lying without contact with other pigs and less ($P < 0.05$) feeding and lying with contact with other pigs compared to CON pigs

Social status did not affect the frequency of behaviors observed on days 1 -3 or 8 – 10 (Table 4-4). However, subordinate pigs showed less ($P < 0.05$) frequent standing/walking behavior than the dominant or intermediate pigs during the treatment period.

Differential white blood cells and plasma haptoglobin concentration. The percentage of differential white blood cells and the neutrophil:lymphocyte (N:L)

Table 4-3. Frequency of behaviors of pigs observed from days 1 to 3 (Treatment; CON at 23 ± 2°C vs HEAT at 33 ± 2°C from day 1 to day 7) and observed from days 8 to 10 (Recovery; CON and HEAT at 23 ± 2°C from day 8 to day 14). Table values are least squares means ± SE.

Measures	Treatment		Recovery	
	CON	HEAT	CON	HEAT
Number of animals	12	12	12	12
Standing/Walking, %	6.7 ± 0.6[a]	5.5 ± 0.6[a]	6.0 ± 0.6[a]	3.9 ± 0.6[b]
Drinking, %	0.9 ± 0.7[c]	5.9 ± 0.7[a]	1.7 ± 0.7[b]	1.6 ± 0.7[bc]
Feeding, %	7.7 ± 0.7[a]	4.6 ± 0.7[b]	6.9 ± 0.7[a]	7.6 ± 0.7[a]
Lying with contact, %	49.9 ± 1.9[b]	42.4 ± 1.9[c]	50.4 ± 1.9[b]	64.3 ± 1.9[a]
Lying without contact, %	34.8 ± 1.8[b]	41.6 ± 1.8[a]	35.1 ± 1.8[b]	22.6 ± 1.9[c]

[a,b,c] Means within a row with uncommon superscripts differ ($P < 0.05$).

Table 4-4. Frequency of behaviors of dominant (Dom.), intermediate (Int.), and subordinate (Sub.) pigs observed from days 1 to 3 (Treatment; CON at 23 ± 2°C vs HEAT at 33 ± 2°C from day 1 to day 7) and observed from days 8 to 10 (Recovery; CON and HEAT at 23 ± 2°C from day 8 to day 14). Table values are least squares means ± SE.

Measures	Treatment			Recovery		
	Dom.	Int.	Sub.	Dom.	Int.	Sub.
Number of animal	8	8	8	8	8	8
Standing/ walking, %	6.6 ± 0.7^{ab}	6.8 ± 0.7^{a}	4.9 ± 0.7^{c}	4.6 ± 0.7^{c}	5.0 ± 0.7^{bc}	5.1 ± 0.7^{bc}
Drinking, %	3.0 ± 0.7^{abc}	3.3 ± 0.7^{ab}	3.9 ± 0.7^{a}	1.9 ± 0.7^{bc}	1.6 ± 0.7^{c}	1.5 ± 0.7^{c}
Feeding, %	6.1 ± 0.6^{c}	6.0 ± 0.6^{bc}	6.3 ± 0.6^{abc}	7.4 ± 0.6^{ab}	7.7 ± 0.6^{a}	6.5 ± 0.6^{abc}
Lying with contact, %	46.1 ± 1.7^{b}	47.6 ± 1.7^{b}	44.8 ± 1.7^{b}	57.3 ± 1.7^{a}	58.4 ± 1.7^{a}	56.3 ± 1.7^{a}
Lying without contact, %	38.2 ± 1.7^{a}	36.2 ± 1.7^{a}	40.2 ± 1.7^{a}	28.8 ± 1.7^{b}	27.2 ± 1.7^{b}	30.6 ± 1.7^{b}

[a,b,c] Means within a row with uncommon superscripts differ ($P < 0.05$).

ratio were not influenced by treatment (Table 4-5). However, the percentage of eosinophils in HEAT pigs increased ($P < 0.05$) from 0.8 ± 0.5 % on day 0 to 2.7 ± 0.5 % on day 7, and then decreased ($P < 0.05$) to 1.2 ± 0.5 % on day 14. In HEAT pigs, plasma haptoglobin concentration increased ($P < 0.05$) from 467 ± 123 ug/ml before treatment to 763 ± 113 ug/ml after a 7-day exposure to elevated temperatures (Table 4-5). CON pigs did not differ in plasma haptoglobin concentration over the three sampling days. HEAT pigs had higher ($P < 0.05$) plasma haptoglobin concentration and percentage of eosinophils than CON pigs on day 7.

There was no difference in differential white blood cell numbers among dominant, intermediate, and subordinate pigs on either day 7 or 14 (Table 4-6). However, intermediate pigs had a higher ($P < 0.05$) percentage of lymphocyte, and lower N:L ratio on day 14 compared to that observed on day 7. Plasma haptoglobin concentration was not affected by social rank (Table 4-6).

Plasma cortisol concentrations. Plasma cortisol concentration in HEAT pigs decreased ($P < 0.05$) from 99.3 ± 8.3 ng/ml before treatment to 85.1 ± 8.3 ng/ml after a 7-day treatment period, and then increased to 110.0 ± 8.3 ng/ml after a 7-day recovery period (Figure 4-1). Plasma cortisol concentration in CON pigs was likewise elevated but not different over the three sampling periods.

Plasma CBG concentration. Plasma CBG concentration in HEAT pigs decreased from 11.4 ± 1.1 μg/ml before treatment to 9.9 ± 1.1 μg/ml after a 7-day treatment, but did not increase after a 7-day recovery period compared to after a

Table 4-5. Plasma haptoglobin concentration and differential white blood cells of pigs before treatment (Before; CON and HEAT at 23 ± 2°C on day 0), after a 7-day treatment (Treatment; CON at 23 ± 2°C vs HEAT at 33 ± 2°C from day 1 to day 7) and after a 7-day recovery period (Recovery; CON and HEAT at 23 ± 2°C from day 8 to day 14). Table values are least squares means ± SE.

	Before		Treatment		Recovery	
Measures	CON	HEAT	CON	HEAT	CON	HEAT
Number of animal	12	12	12	12	12	12
Lymphocyte, %	69.2 ± 2.4	69.2 ± 2.4	69.0 ± 2.4	67.5 ± 2.4	71.4 ± 2.4	68.3 ± 2.4
Neutrophil, %	25.6 ± 2.3	27.3 ± 2.3	27.9 ± 2.3	28.4 ± 2.3	25.4 ± 2.3	28.3 ± 2.3
Monocyte, %	3.4 ± 0.6^{a}	2.8 ± 0.6^{ab}	1.9 ± 0.6^{ab}	1.4 ± 0.6^{b}	2.2 ± 0.6^{ab}	2.3 ± 0.6^{ab}
Eosinophil, %	1.8 ± 0.5^{ab}	0.8 ± 0.5^{b}	1.2 ± 0.5^{b}	2.7 ± 0.5^{a}	1.0 ± 0.5^{b}	1.2 ± 0.5 ^{b}
Neutrophil: Lymphocyte	0.38 ± 0.06	0.42 ± 0.06	0.42 ± 0.06	0.43 ± 0.06	0.37 ± 0.06	0.46 ± 0.06
Haptoglobin (ug/ml)	611 ± 123^{ab}	467 ± 123^{b}	355 ± 108^{b}	763 ± 113^{a}	507 ± 118^{ab}	550 ± 108^{ab}

^{a,b} Means within a row with uncommon superscripts differ ($P < 0.05$).

Table 4-6. Plasma haptoglobin concentration and differential white blood cells of dominant (Dom.), intermediate (Int.), and subordinate (Sub.) pigs before treatment (Before; CON and HEAT at 23 ± 2°C on day 0), after a 7-day treatment (Treatment; CON at 23 ± 2°C vs HEAT at 33 ± 2°C from day 1 to day 7) and after a 7-day recovery period (Recovery; CON and HEAT at 23 ± 2°C from day 8 to day 14). Table values are least squares means ± SE.

Measures	Before			Treatment			Recovery		
	Dom.	Int.	Sub.	Dom.	Int.	Sub.	Dom.	Int.	Sub.
Number of animal	8	8	8	8	8	8	8	8	8
Lymphocyte, %	70.0±2.9^{ab}	68.5±2.9^{ab}	67.0±2.9a^{b}	68.0±2.9^{ab}	66.9 ± 2.9^{b}	69.9 ± 2.9^{ab}	66.9 ± 2.9^{ab}	72.3 ± 2.9^{a}	70.5±2.9^{ab}
Neutrophil, %	25.8±2.8^{ab}	26.8±2.8^{ab}	26.8 ±2.8^{ab}	27.9 ±2.8^{ab}	30.3 ± 2.8^{a}	26.4 ± 2.8^{ab}	30.5 ± 2.8^{ab}	24.8 ± 2.8^{b}	25.3±2.8^{ab}
Monocyte, %	0.8± 0.8^{d}	3.8 ± 0.8^{ab}	4.9 ± 0.8^{a}	2.4 ± 0.8^{bcd}	1.4 ± 0.8^{bcd}	1.3 ± 0.8^{cd}	1.8 ± 0.8^{bcd}	1.8 ± 0.8^{bcd}	3.1 ±0.8^{abc}
Eosinophil, %	1.5 ± 0.6	1.0 ± 0.6	1.5 ± 0.6	1.8 ± 0.6	1.5 ± 0.6	2.5 ± 0.6	0.9 ± 0.6	1.3 ± 0.6	1.1 ± 0.6
Neutrophil : Lymphocyte	0.4±0.1^{ab}	0.4±0.1^{ab}	0.4±0.1^{ab}	0.4±0.1^{ab}	0.5±0.1^{a}	0.4±0.1^{ab}	0.5±0.1^{ab}	0.4±0.1^{b}	0.4±0.1^{ab}
Haptoglobin (ug/ml)	532 ±128	660 ±128	426 ± 177	604 ±137	523 ± 128	551 ± 128	587 ± 128	403 ± 137	596 ± 137

^{a,b,c,d} Means within a row with uncommon superscripts differ (P < 0.05).

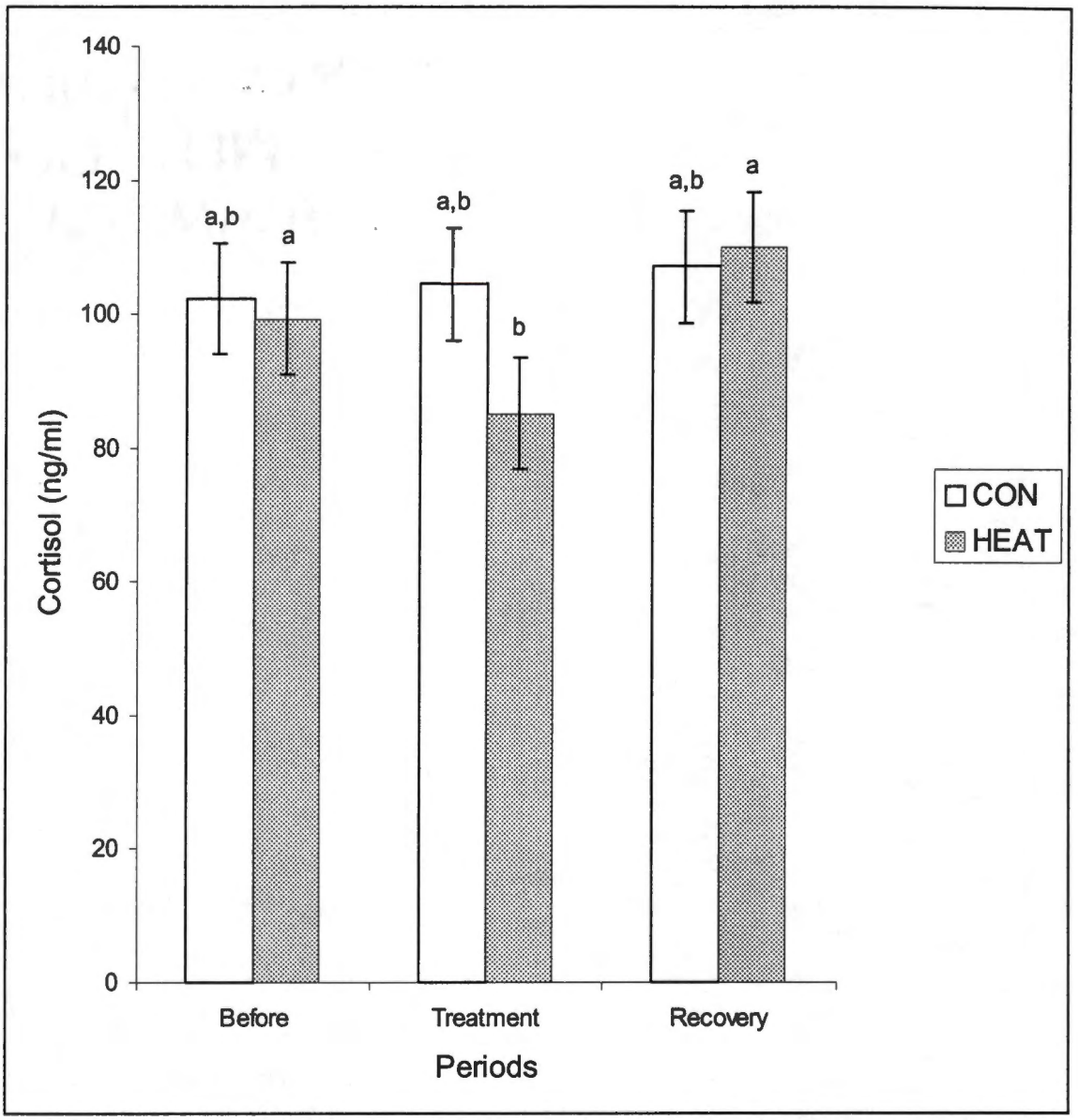


Figure 4-1. Plasma cortisol concentration of pigs before treatment (Before; CON and HEAT at 23 ± 2°C onday 0), after a 7-day treatment (Treatment; CON at 23 ± 2°C vs HEAT at 33 ± 2°C from day 1 to day 7) and after a 7-day recovery period (Recovery; CON and HEAT at 23 ± 2°C from day 8 to day 14). Each column represents the mean ± SE (n = 12). Least squares means with a different letter differ ($P < 0.05$).

7-day treatment (Figure 4-3). Plasma CBG concentration in CON pigs was not different over the three sampling periods.

Hepatic CBG mRNA expression levels. In both of CON pigs and HEAT pigs, there was no significant difference in hepatic CBG mRNA expression over the three sampling periods (Figure 4-5). There was also no difference in hepatic CBG mRNA expression between CON pigs and HEAT for each period.

There was no difference in plasma cortisol (Figure 4-2) and CBG concentrations (Figure 4-4), and hepatic CBG mRNA expression levels (Figure 4-6) due to social rank.

Discussion

Although there was no significant difference in body weight gain between CON pigs and HEAT pigs, the rate of gain in HEAT pigs was significantly lower during the 7-day treatment compared to the 7-day recovery period. Morrow-Tesch et al. (1994) found that differences in body weight between heat-stressed pigs and non-stressed pigs increased as time progressed and was significant at least 14 days after exposure to heat stress. A 4 hour-acute heat stress treatment was not sufficient to cause weight change in the young pig (Hicks et al., 1998). Therefore, the lack of difference in body weight between CON and HEAT pigs seen here may be a result of the short duration of heat treatment as well.

A significant difference in body weight as affected by social status was observed after a 7-day treatment period but disappeared after a 7-day recovery

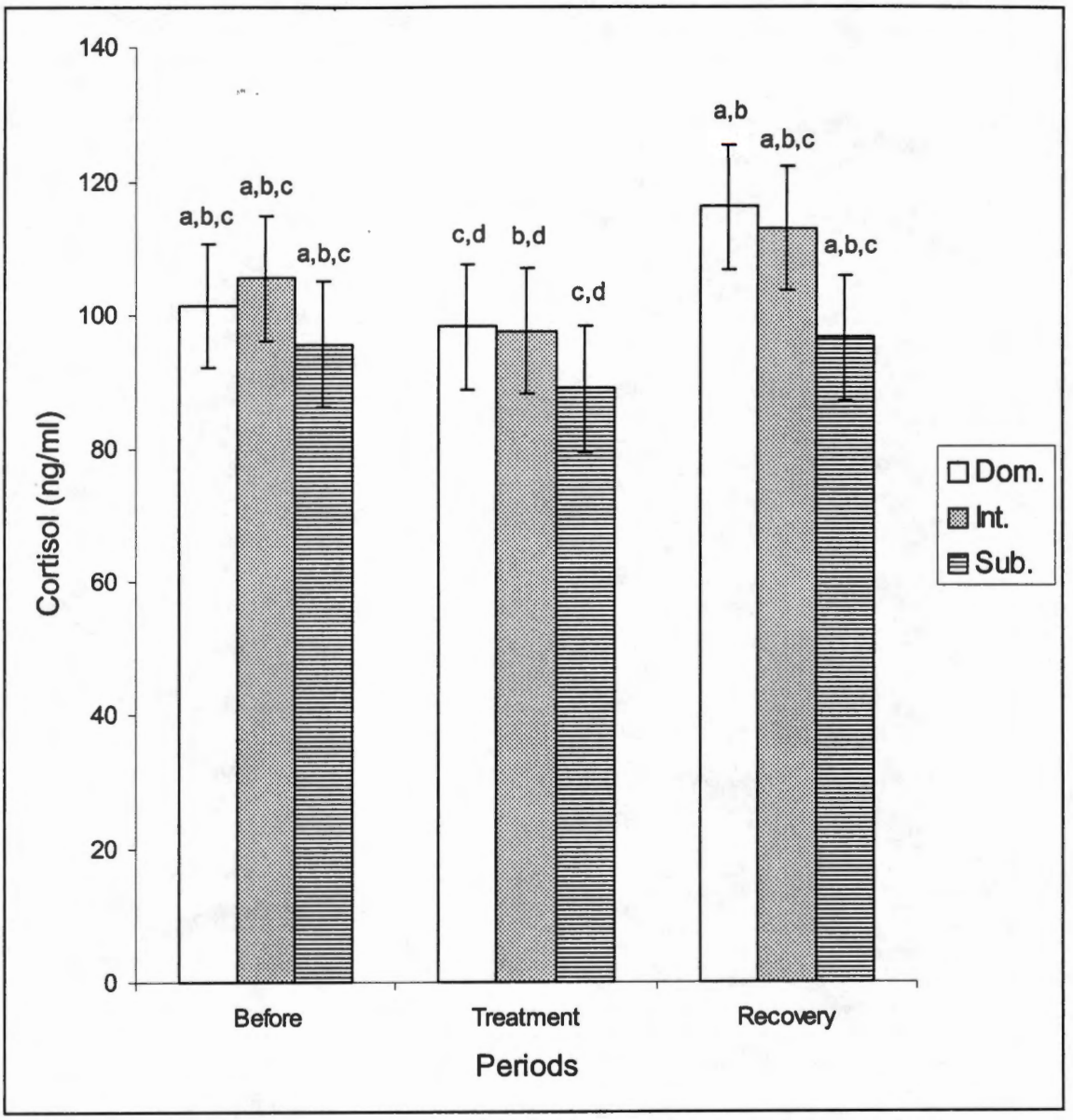


Figure 4-2. Plasma cortisol concentration of dominant (Dom.), intermediate (Int.), and subordinate (Sub.) pigs before treatment (Before; CON and HEAT at 23 ± 2°C on day 0), after a 7-day treatment (Treatment; CON at 23 ± 2°C vs HEAT at 33 ± 2°C from day 1 to day 7) and after a 7-day recovery period (Recovery; CON and HEAT at 23 ± 2°C from day 8 to day 14). Each column represents the mean ± SE (n = 8). Least squares means with a different letter differ ($P < 0.05$).

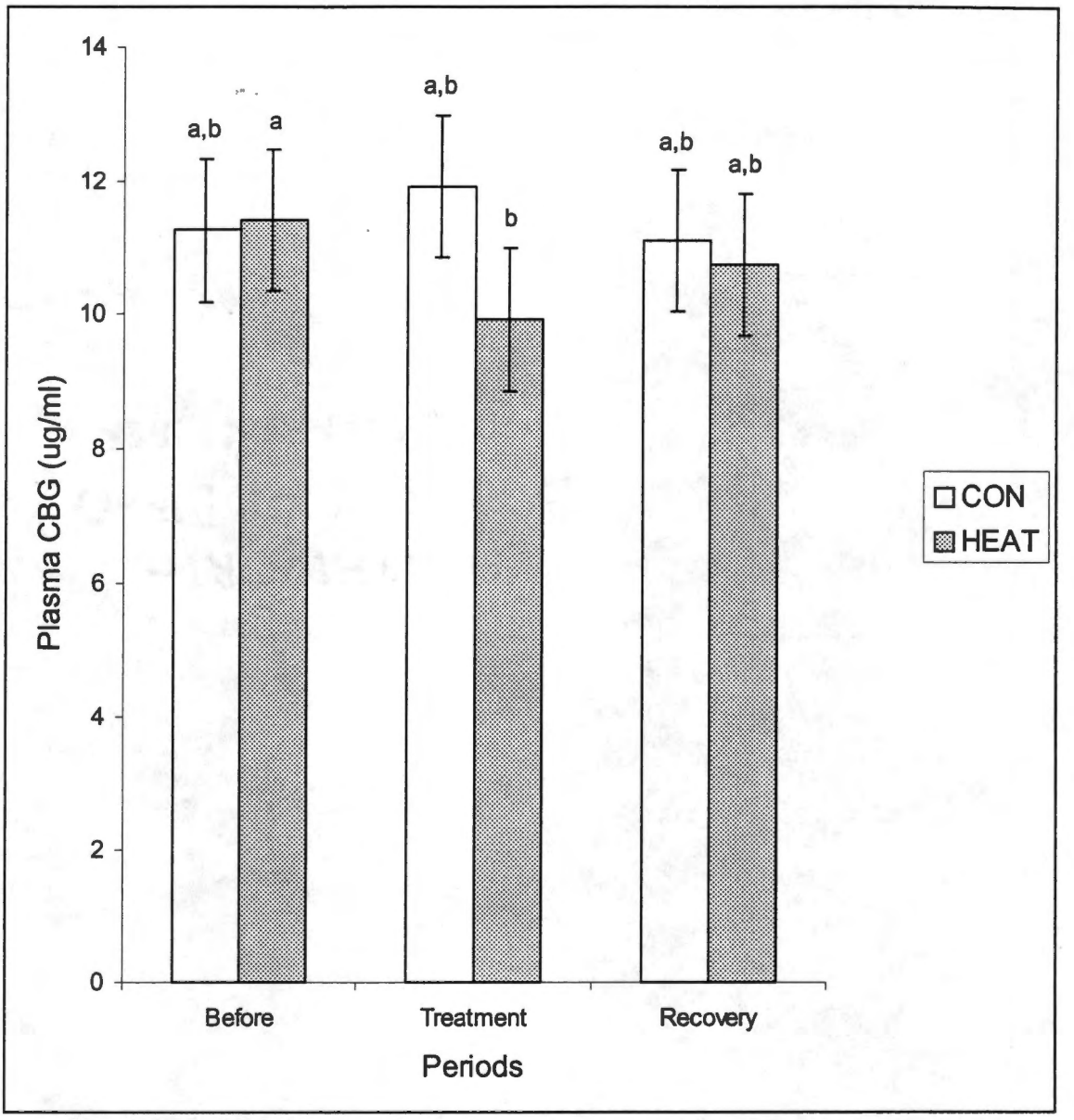


Figure 4-3. Plasma CBG concentration of pigs before treatment (Before; CON and HEAT at 23 ± 2°C on day 0), after a 7-day treatment (Treatment; CON at 23 ± 2°C vs HEAT at 33 ± 2°C from day 1 to day 7) and after a 7-day recovery period (Recovery; CON and HEAT at 23 ± 2°C from day 8 to day 14). Each column represents the mean ± SE (n = 12). Least squares means with a different letter differ ($P < 0.05$).

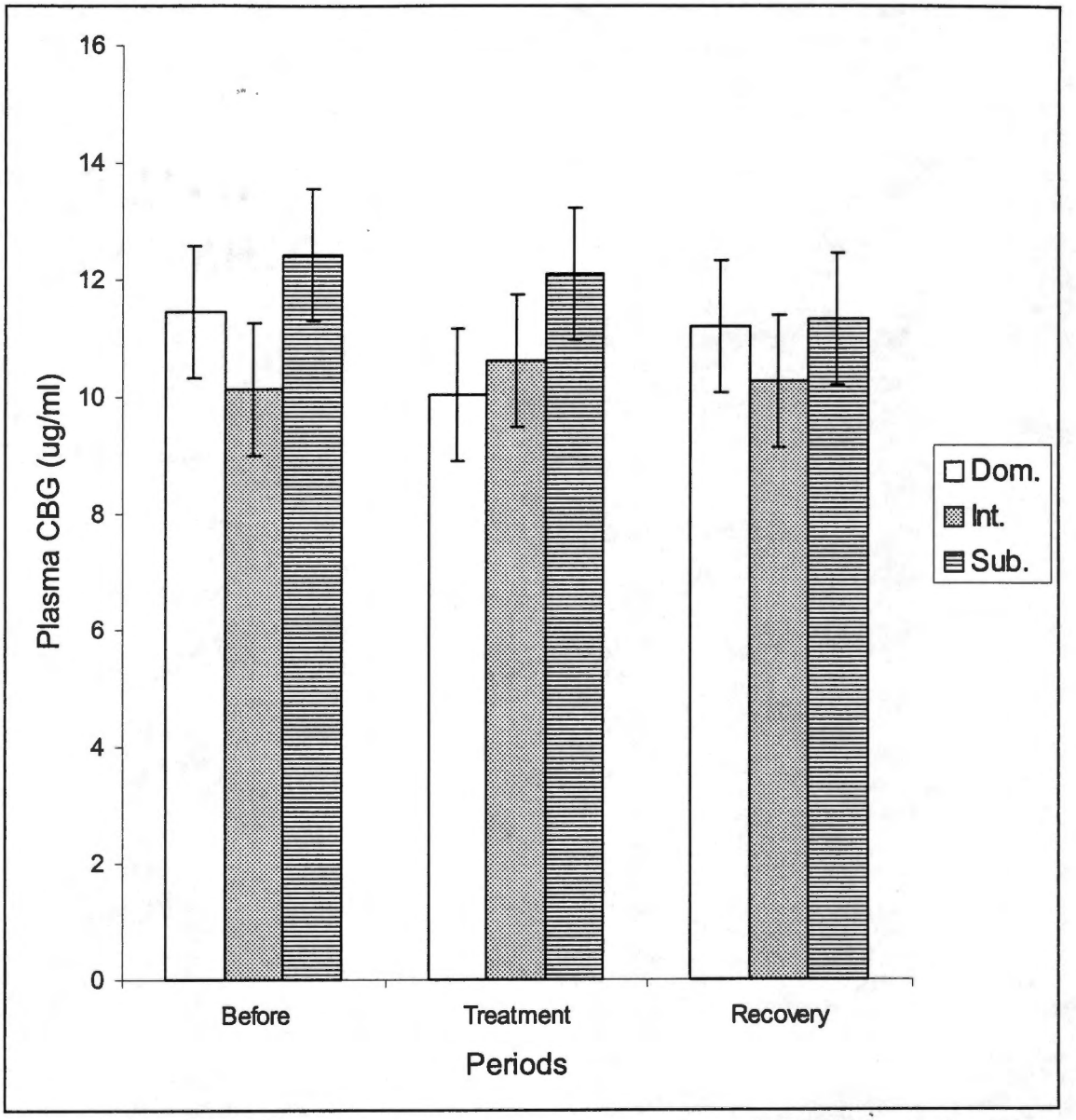


Figure 4-4. Plasma CBG concentration of dominant (Dom.), intermediate (Int.), and subordinate (Sub.) pigs before treatment (Before; CON and HEAT at 23 ± 2°C on day 0), after a 7-day treatment (Treatment; CON at 23 ± 2°C vs HEAT at 33 ± 2°C from day 1 to day 7) and after a 7-day recovery period (Recovery; CON and HEAT at 23 ± 2°C from day 8 to day 14). Each column represents the mean ± SE (n = 8).

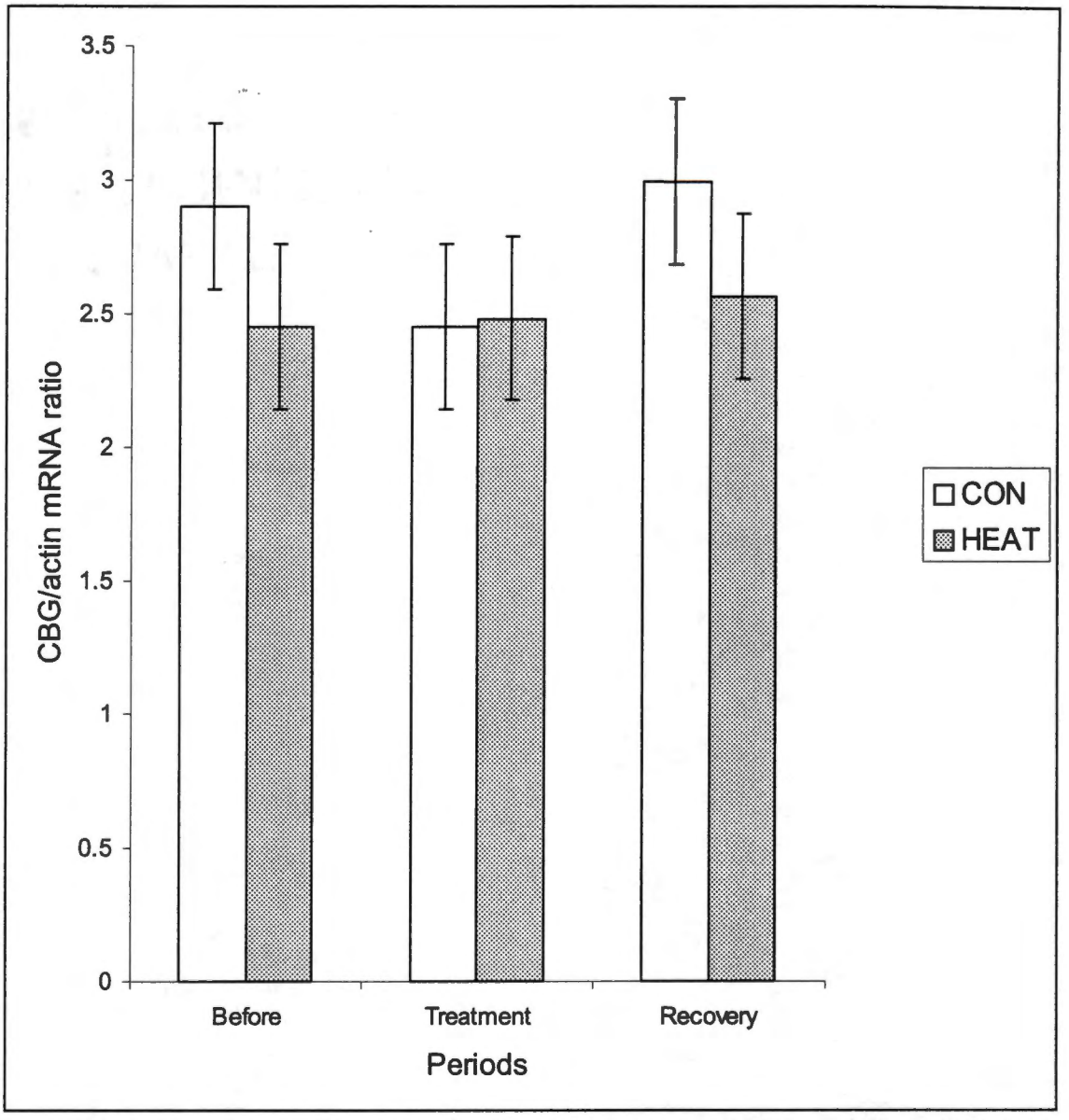


Figure 4-5. Hepatic CBG/actin mRNA ratio of pigs before treatment (Before; CON and HEAT at 23 ± 2°C on day 0), after a 7-day treatment (Treatment; CON at 23 ± 2°C vs HEAT at 33 ± 2°C from day 1 to day 7) and after a 7-day recovery period (Recovery; CON and HEAT at 23 ± 2°C from day 8 to day 14). Each column represents the mean ± SE (n = 12).

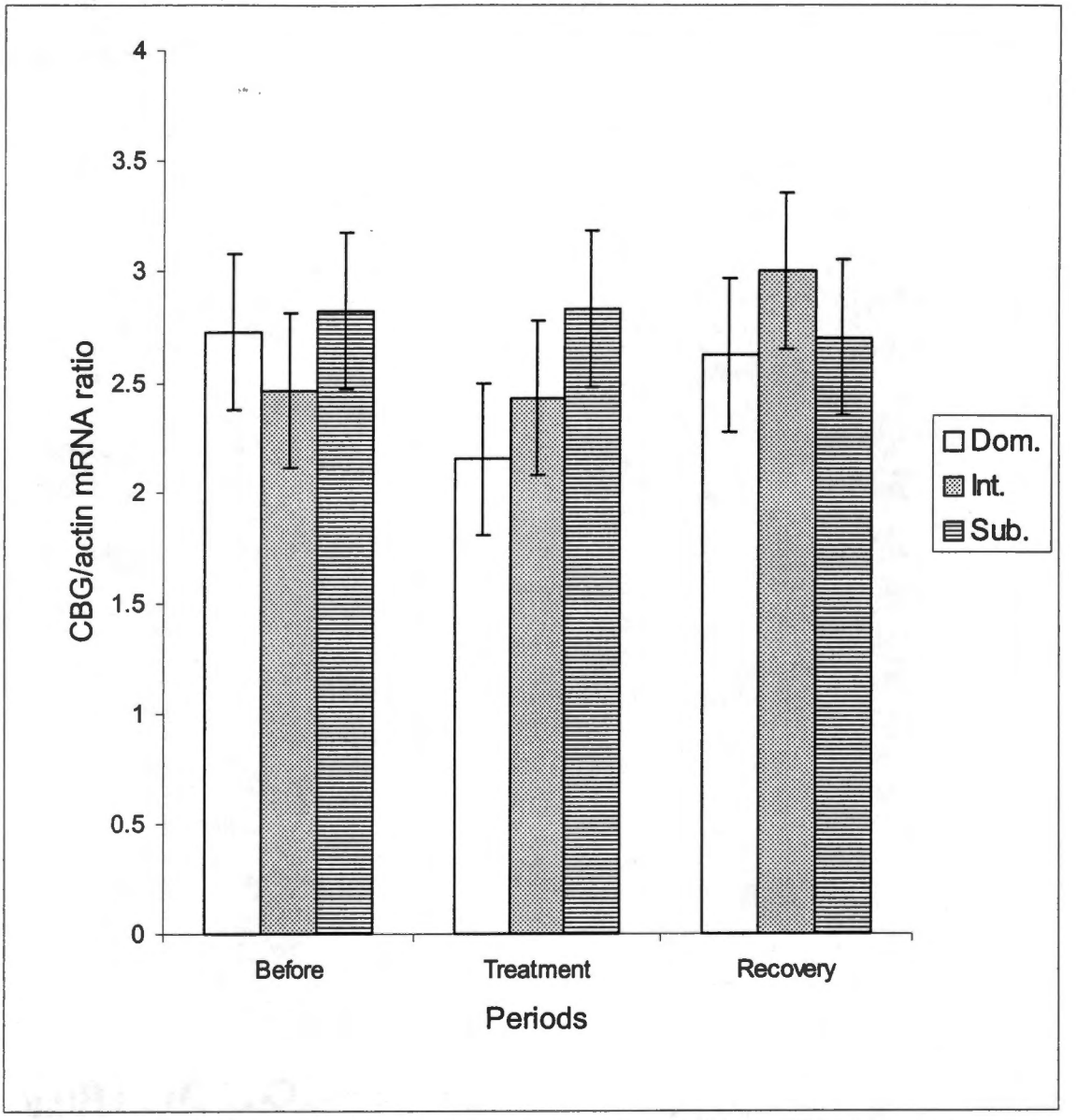


Figure 4-6. Hepatic CBG/actin mRNA ratio of dominant (Dom.), intermediate (Int.), and subordinate (Sub.) pigs before treatment (Before; CON and HEAT at 23 ± 2°C on day 0), after a 7-day treatment (Treatment; CON at 23 ± 2°C vs HEAT at 33 ± 2°C from day 1 to day 7) and after a 7-day recovery period (Recovery; CON and HEAT at 23 ± 2°C from day 8 to day 14). Each column represents the mean ± SE (n = 8).

period. Morrow-Tesch et al. (1994) reported that the heaviest pig usually became the dominant pig and that differences in body weight between dominant and submissive pigs increased as time progressed. However, in the present study, subordinate pigs were heavier than dominant pigs on day 7 while there was no difference in body weight on day 0. An increase in the rate of gain from day 7 to 14 was greater in dominant pigs than in intermediate and subordinate pigs. This data suggests that the body weight of the pig may not affect the establishment of social rank, but social status may influence the rate of weight gain as time progresses.

Heat stress influenced the frequency of behaviors of pigs. Hicks et al. (1998) reported that pigs exposed to a 4-hour acute heat stress spent less time feeding and standing but more time lying. Reduced body weight gain during heat treatment may be due to suppressed feed intake (McGlone et al., 1987). In the present study, a 7-day heat treatment resulted in pigs showing a greater frequency of drinking and lying without contact and reduced frequency of feeding and lying with contact. Decreased feeding frequency during the heat stress may have contributed to their suppressed rate of gain. Effect of heat stress on pig behaviors measured here disappeared after a 7-day recovery period.

Social status did not affect the frequency of behaviors in pigs. Similarly, Hicks et al. (1998) reported that pig behavior was not influenced by social status, but greatly influenced by cold, heat, or shipping stress.

Heat stress increased the percentage of eosinophils and plasma haptoglobin concentration, but did not affect the percentage of the other blood

cells. Similarly, Morrow-Tesch et al. (1994) found that there was no effect of heat stress for measures of immunity, but the number of eosinophils increased following chronic stress. Plasma concentration of haptoglobin in pigs injected with turpentine increased two fold by the second day and then returned to pre-treatment levels by day 8 post treatment (Eckersall et al., 1996). In contrast, Hicks and coworkers (1998) reported that a 4-hour acute heat stress did not affect the numbers of differential white blood cells or plasma haptoglobin concentration in pigs. Therefore, the effect of heat stress on the differential white blood cells and plasma haptoglobin concentration may be related to the duration of heat stress.

McGlone et al. (1993) found that natural killer cell cytotoxicity was suppressed among intermediate pigs as compared to dominant pigs after a 4-hour shipping experience, and that the effect of social status on natural killer cell activity may have been influenced by the shipping stress. Morrow-Tesch et al. (1994) reported that socially intermediate pigs maintained at 24 ± 2 °C had lower neutrophil numbers compared to pigs subjected to high air temperature (33 ± 2 °C). Lymphocyte proliferation was lower in intermediate pigs than in the other pigs during heat stress but similar among social status during cold stress (Hicks et al., 1998). In the present study, since intermediate pigs had a higher percentage of neutrophils and neutrophil:lymphocyte ratio on day 7 as compared to day 14, the effect of social stress on differential white blood cell number may be more significant in intermediate pigs than in dominant or subordinate pigs.

Plasma cortisol concentration decreased in response to a chronic heat stress and returned to the initial level after a 7-day recovery period. Gilts exposed to 8 days of high temperature (32 °C) exhibited a decrease in plasma cortisol level, and an increase in plasma ACTH level (Marple et al., 1972). The authors suggested that the decrease in plasma cortisol levels while plasma ACTH levels increased may be due to decreased adrenal responsiveness to ACTH and/or increased turnover of plasma cortisol. Becker et al. (1985) found that gilts subjected to a 6-hour heat stress for three consecutive days had increased plasma cortisol concentrations, but overall plasma cortisol levels decreased from day 1 to day 3. These authors suggested that the decrease in plasma cortisol concentration over the three 3 days may have resulted from a possible alteration in metabolism of cortisol by the liver. In the present study, the decrease in plasma cortisol concentration following a 7-day heat treatment may similarly be due to increased metabolism and/or utilization of cortisol to cope with the stressful condition.

There was no significant effect of social status on plasma cortisol concentration in the present study. Parrott and Misson (1989) found that salivary cortisol levels in pigs significantly increased three hours after mixing. However, Ekkel et al. (1997) measured the salivary cortisol levels in young pigs 6 and 41 days after mixing to examine whether chronic social conflicts would occur with elevated levels of cortisol. They found that the salivary cortisol levels were not different between unmixed pigs and mixed pigs on either of the two days examined. McGlone (1986) suggested that social stability within a group of pigs

may be observed within 24 hours after mixing, and social stress is presumed only to be present during the first 24 hours after mixing. In the present study, therefore, no difference in plasma cortisol concentration among pigs in different social rank may indicate that the changes in cortisol level may depend on the duration of social stress.

Chronic heat stress for 7 days decreased plasma CBG concentration in the present study. Similarly, Dalin and coworkers (1993) found that plasma CBG levels in pigs decreased after surgery, but were not affected by relocation or grouping. In the rat, starvation for 2 days and restriction for 2 hours decreased plasma CBG levels, but acute stress by ether treatment did not affect plasma CBG levels (Tinnikov, 1993). In contrast, Hicks and coworkers (1998) reported that acute heat, cold, or shipping stresses did not affect plasma CBG levels in the pig. In the present study, plasma CBG concentration returned to initial levels after a 7-day recovery period. It would appear that plasma CBG levels may be influenced by duration and/or type of stress. From the result of the present study, the decrease in plasma CBG may be associated with the decrease in plasma cortisol level because plasma CBG level influences the metabolic clearance rate of cortisol in circulation (Bright, 1995).

It has not been demonstrated directly whether changes in plasma CBG by stress result from changes in CBG production, metabolic degradation and/or transfer to target tissues. There is indirect evidence regarding the mechanism for the decrease in CBG following stress. Dalin and coworkers (1993) explained that the decrease in plasma CBG levels after surgery may be due to increased

cleavage of CBG by neutrophil elastase. Stefanski (2000) found a marked decrease in plasma CBG levels with increased number of granulocyte but no change in total corticosterone levels in subordinate rats after chronic confrontation. The author suggested that the reduction of plasma CBG levels may result from increased degradation of CBG by neutrophil elastase on the surface of neutrophil granulocytes rather then decreased CBG production. However, an inhibition in hepatic CBG biosynthesis has been also suggested as a possible mechanism for the decrease in CBG following stress (Fleshner et al., 1995; Bernier et al., 1998). There is little evidence about the effect of stress on hepatic CBG mRNA expression. Previous studies have found that glucocorticoids inhibit the hepatic biosynthesis of CBG in the adult rat (Smith and Hammond, 1992) and sheep (Berdusco et al., 1993). One would expect that reduced cortisol levels within the circulation might reduce its inhibitory effects on CBG synthesis in the liver. In the present study, although plasma cortisol levels decreased, hepatic CBG mRNA expression in pigs was not changed after 7 days of exposure to 33 ± 2 °C followed by 7 days exposure to 23 ± 2 °C. Therefore, the decrease in plasma CBG levels after a 7-day heat treatment may be a result of other factors such as increased metabolism and/or transfer to extravascular space rather than a decrease in CBG biosynthesis by the liver.

As reported here, social status did not affect plasma CBG levels or hepatic CBG expression levels in pigs. However, when pigs were exposed to other stressors including cold, heat, or shipping after the establishment of social status, there was no difference in plasma CBG levels among dominant, intermediate,

and subordinate pigs (Hicks et al., 1998). In the rat, chronic social stress resulted in reduced plasma CBG levels in both dominant and subordinate animals (Spencer et al., 1996). In horses, CBG binding capacity decreased slightly without changing total cortisol in social stress situations (Alexander and Irvine, 1998). In the present study, the lack of differences in plasma cortisol and CBG levels among dominant, intermediate, and subordinate pigs during heat treatment may be related to the interactive effect of heat and social stress (Morrow-Tesch et al., 1994).

In conclusion, growth rate, behavioral, and physiological measures in pigs were significantly affected by chronic heat stress but not by social stress. The reduced circulating levels of cortisol and CBG in pigs following 7 days exposure to elevated temperature may not be determined by hepatic CBG mRNA expression.

CHAPTER 5. GENERAL DISCUSSION

It has been reported that plasma CBG level influences the kinetics of cortisol (Bright, 1995). Because of this, plasma CBG level modulates the bioavailabilty of cortisol in tissue (Rosenthal et al., 1969). Plasma CBG levels have been measured under different physiological conditions (Seralini et al., 1990; Smith and Hammond, 1991; Dalin et al, 1993); however, it is still unclear whether the changes in plasma CBG under different conditions is attributed to an alteration in biosynthesis of CBG or other factors including metabolic clearance and transfer to the extravascular space. Understanding how plasma CBG level is determined may be helpful for predicting the bioavailability and distribution of cortisol in circulation under various physiological conditions.

Developing a 500 bp partial porcine CBG cDNA made it possible to probe CBG mRNA levels in several tissues in pigs and then demonstrate that the liver appears to be the primary source of CBG in the postnatal pig. This is an important finding because it indicates that CBG synthesis in liver may be related to changes in plasma CBG level. The partial porcine CBG cDNA can be used to measure hepatic CBG mRNA expression levels in pigs, and also clone the full length of CBG cDNA that may provide information regarding the regulation of CBG biosynthesis. Comparison of the deduced primary structure of the partial porcine CBG with that of human (Hammond et al., 1987), sheep, rabbit, and rat CBG cDNA sequence revealed that there are several regions well conserved regarding steroid binding and glycosylation (Hammond et al., 1991).

Determination of relationships between plasma CBG level and hepatic CBG biosynthesis levels during development may provide important information regarding the bioavailability and conservation of cortisol in the prenatal and postnatal pig. In the present study, a decline in fetal pig hepatic CBG mRNA from mid- to late gestation with a concomitant decrease in plasma CBG levels, was similar to the results reported previously in the fetal rabbit (Seralini et al., 1990) and fetal rat (Smith and Hammond, 1991). A decrease in plasma CBG levels due to a reduction in hepatic CBG biosynthesis may lead to increased free cortisol levels towards term that regulate dramatic maturational events in the fetus (Liggins, 1994).

The present study found that the pattern of increasing plasma CBG level during postnatal development did not agree with the pattern of increasing hepatic CBG mRNA expression level. An earlier study also reported an age-related difference in the profiles of rat hepatic CBG mRNA and plasma CBG concentrations, suggesting the difference may be attributed to differences in the clearance rate of CBG associated with age (Smith and Hammond, 1991). Therefore, plasma CBG levels in pigs during the postnatal period may be determined by hepatic CBG biosynthesis as well as other factors such as metabolic degradation or transfer to extravascular space. The increased plasma CBG level during the postnatal period may have an important role as a relatively inert reservoir in which cortisol is protected from metabolism and excretion, but still available by dissociation to provide sufficient cortisol quickly under emergency situations such as stress (Ballard, 1979).

Chronic heat stress had significant effects on behavioral and physiological responses in pigs, especially plasma CBG and haptoglobin levels. Following a 7-day exposure to elevated air temperature (33 ± 2°C), plasma cortisol and CBG concentrations decreased. However, hepatic CBG mRNA expression level was not affected by the chronic heat stress. Although it has been demonstrated that acute stress in rats either had no effect on plasma CBG levels (Tinnikov (1993) or increased plasma CBG levels (Bassett, 1996; Nyberg et al., 1988), chronic stress generally results in decrease in plasma CBG concentration (Tinnikov, 1993; Fleshner et al., 1995). There is no direct evidence regarding whether plasma CBG level is determined by its biosynthesis or other factors during stress. There is indirect evidence indicating that a reduction in plasma CBG level during stress could be a result of increased degradation of CBG (Dalin et al., 1993; Fleshner et al., 1995; Spencer et al., 1996). In the present study, plasma CBG levels in young pigs subjected to elevated temperatures decreased without a corresponding change in hepatic CBG mRNA levels. Therefore, plasma CBG level during chronic heat stress may not be determined by hepatic CBG mRNA expression, but instead other factors such as increased degradation and/or transfer to extravascular space.

It may be concluded from the results of the present study that liver appears to be the primary source of CBG biosynthesis in the postnatal pig. In pigs, circulating CBG levels may be determined mainly by hepatic CBG biosynthesis during the prenatal period and by hepatic CBG biosynthesis as well as other factors during the postnatal period. However, changes in plasma CBG

levels in pigs following exposure to elevated temperatures do not appear to be a result of hepatic biosynthesis based on CBG mRNA levels. Plasma CBG levels in pigs may be determined differently according to stage of development or various physiological conditions including stress. An understanding of the relationship between plasma CBG and hepatic CBG mRNA levels under various physiological conditions may provide important fundamental information that will aid in understanding the mechanism determining plasma CBG levels and bioavailability of cortisol in circulation.

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APPENDICES

Appendix A. Procedure for total RNA extraction

A. Procedure

1. Homogenize approximately 200 mg of tissue with Tissumizer (Tekmar Company, Cininnati, OH) in 3 ml of Tri-Reagent solution using 5 to 6 strokes.
2. Centrifuge the homogenate at 12,000 x g for 10 min at 4°C to remove the insoluble material.
3. Transfer the clear supernatant to a tube and allow the sample to stand for 5 min at room temperature.
4. Add 0, 6 ml of chloroform (0.2 ml per ml of Tri-Reagent) and cover the tube tightly with cap.
5. Vortex the mixture vigorously for 20 sec and allow to stand for 10 min at room temperature.
6. Centrifuge the resulting mixture at 12,000 x g for 15 min at 4°C.
7. Transfer the upper aqueous phase to a new tube.
8. Add 2 ml of isoprophanol (0.5 ml per ml of Tri-Reagent) to the aqueous phase and vortex briefly.
9. Incubate the sample at –70 °C overnight.
10. Centrifuge the sample at 12,000 x g for 10 min at 4°C and discard the solution.

11. Add 4 ml of 75 % ethanol to the pellet and vortex vigorously to wash out salts.
12. Centrifuge the sample at 12,000 x g for 5 min at 4°C and discard the solution.
13. Repeat steps 11 and 12.
14. Add 1 ml of 75% ethanol to the pellet and transfer the mixture to the RNAse free-microcentrifuge tube.
15. Transfer 100 ml of the mixture to a RNAse free-microcentrifuge tube and centrifuge it at 12,000 x g for 10 min at 4°C.
16. Discard the solution and dry the pellet at room temperature.
17. Add 30 ul of 0.1 mM EDTA to the dried pellet.
18. Dissolve the pellet at 65 °C for 10 min and chill it on ice.
19. Dillute 5 ul of the RNA solution with 1 ml water (1/200).
20. Measure its absorbance at 260 nm to determine the concentration of the RNA solution.

Appendix B. Procedure for Northern blot analysis

A. Preparation of reagents

1. 5 x formaldehyde gel-running buffer

   0.1 M MOPS (3-N-morpholinopropanesulfonic acid, pH 7.0)

   40 mM sodium acetate

   5 mM EDTA (pH 8.0)

   - Dissolve 10.3 g of MOPS in 400 ml of 50 mM sodium acetate [6.7 ml 3 M sodium acetate (pH 5.2) + 393.3 ml distilled water treated with 0.1% DEPC]
   - Adjust the solution to pH 7.0 with 2 N NaOH.
   - Add 5 ml of 0.5 M EDTA (pH 8.0) treated with 0.1 % DEPC
   - Adjust the final volume to 500 ml with distilled water treated with 0.1 % DEPC.
   - Sterilize the solution by filtration through a 0.2 um filter and store it at room temperature protected from light

2. 1.3 % agarose gel containing formaldehyde

   - Melt 1.3 g of agarose in 76 ml of water treated with 0.1% DEPC and cool it to 60 °C.
   - Add 24 ml of 5 x formaldehyde gel-running buffer and 22 ml of formaldehyde (final 1 x and 2.2 M, respectively).

3. 20 x SSPE-DEPC

- Dissolve 175.3 g of NaCl, 27.6 g of NaH2PO4.H2O and 7.4 g of EDTA in 800 ml of distilled water.
- Adjust the pH to 7.4 with 10 N NaOH.
- Adjust the volume to 1 liter with distilled water.
- Add 1 ml of DEPC solution.
- Stir well and stand overnight at room temperature.
- Sterilize by autoclaving and store at room temperature

4. 50 x Denhardt's solution

5 g Ficoll (Type 400, Pharmacia)

5 g polyvinylpyrrolidone

5 g bovine serum albumin (Pentex Fraction V)

- Dissolve deionized water to 500 ml
- Filter and stored at -20°C

5. Prehybridization solution

5x SSPE-DEPC	5 ml of 20x SSPE-DEPC soltuion
50% formamide	10 ml of deionized formamide
water-DEPC	4 ml
2x Denhardt's	0.8 ml of 50x Denhardt's solution
0.2% SDS	0.2 ml of 20% SDS solution

Yeast total RNA(300 ug/ml) 6 mg

B. Procedure

1. Prepare the loading sample as described below:

	Sample tube	Dye tube
RNA solution (up to 30 g)	4.5 ul	none
5 x formaldehyde gel-running buffer	2.0 ul	2.0 ul
Formaldehyde	3.5 ul	3.5 ul
Formamide	10.0 ul	10.0 ul
Water with 0.1% DEPC	none	2.5 ul
Loading dye	none	2.0 ul
Final volume of loading sample	20.0 ul	20.0 ul

2. Incubate the loading sample for 15 min at 65°C.
3. Chill it on ice and then microcentrifuge briefly.
4. Run the gel in 600 ml of 1 x formaldehyde gel-running buffer (200 ml of 5 x formaldehyde gel-running buffer + 782 water with 0.1% DEPC + 18 ml formaldehyde) for 5 min at 70 voltage (5 voltage/cm of distance between cathode and anode) before loading the samples.
5. Immediately load the samples into the well of the gel.
6. Run the gel at 70 voltage.

7. The buffer from each reservoir should be collected, mixed and returned to the gel apparatus every 1 - 2 hr.
8. After electrophoresis is completed, add 60 ul of ethidium bromide solution (10 mg/ml) in 600 ml of gel-running buffer and diffuse well by gently mixing for 30 min at room temperature.
9. Examine the gel on an ultravilolet transilluminator to ensure the quality of total RNA and equivalent loading among samples.
10. Destain the gel in 300 ml of 0.1 N ammonium acetate treated with 0.1% DEPC for 30 min at room temperature.
11. Repeat step 10 three times until the dye on the gel disappears.
12. Soak the destained gel in 300 ml of 10 x SSPE solution treated with 0.1% DEPC.
13. Transfer the total RNA from the gel to nylon transfer membrane in 10x SSPE solution treated with 0.1% DEPC using Turboblotter system (Scheicher & Schuell, Keene, NH) overnight at room temperature.
14. After transferring, cross-link the blot in UV crosslinker (1,200 kJ/m^2, Hoefer scientific instruments, San Francisco, CA) and store it at -20°C.
15. Incubate the blot in prehybridization solution at 42°C overnight.
16. Label porcine CBG cDNA probe with 60 uCi of [α-^{32}P] dCTP using a random primer labeling kit (Stratagene, La Jolla, CA).
17. Remove unreacted nucleotide through column containing 5% Sephadex G-50.

18. Count the radioactivity of the ^{32}P-labeled CBG cDNA probe and calculate the volume for 4 x 10^7 cpm.
19. Denature the ^{32}P-labeled CBG cDNA probe by heating at 95°C for 10 min and then add it to the prehybridization solution (2 x 10^6 cpm/ml).
20. Incubate the blot in the hybridization solution at 42°C overnight.
21. Wash the blot twice with 500 ml of 2 x SSPE containing 0.1% SDS at room temperature for 5 min.
22. Wash the blot once with 500 ml of 1 x SSPE containing 0.1% SDS at room temperature for 10 min
23. Wash the blot once with 500 ml of 1 x SSPE containing 0.1% SDS at 65°C for 10 min.
24. Wash the blot once with 500 ml of 0.1 x SSPE containing 0.1 % SDS at 65°C for 20 min.
25. Expose the blot to Kodak X-Omat film at -70°C for 24 hr to detect the radioactivity exposed from the radiolabeled porcine CBG probe.
26. For reprobing the blot with human β-actin cDNA, strip off the porcine CBG cDNA probe by boiling the blot in 600 ml of 0.2 % SDS treated with 0.1% DEPC for 20 min, and then repeat step 15 through 25.
27. Radioactivity is measured using an UltraScan XL Laser Densitometer (LKB Produkter AB, Bromma, Sweden).
28. The expression level of porcine CBG mRNA is expressed relative to β-actin mRNA level.

Appendix C. Procedure for radioimmunoassay (RIA)

A. Procedure

1. Label four plain (uncoated) 12 x 75 mm polypropylene tubes with total count (T) and nonspecific binding (NSB) in duplicate.
2. Label the cortisol antibody tubes in duplicate with the calibrators A (maximum binding, 0 ug/dl), B (0.5 ug/dl), C (1 ug/dl), D (5 ug/dl), E (10 ug/dl), F (20 ug/dl), cortisol standard (human serum), CON(a pool of pig serum), and sample numbers.
3. Pipet 25 ul of the zero calibrator A into the NSB and A tubes.
4. Pipet 25 ul of each remaining calibrators, control, and sample into its appropriate tube.
5. Add 1 ml of [^{125}I] cortisol to every tube and vortx.
6. Incubate the tubes in a water bath at 37°C for 45 min.
7. Decant the contents of all tubes except the total count tubes (T) thoroughly using a foam decanting rack.
8. Allow them to drain for 2 min and then strike the tubes on paper towel to shake off all residual droplets.
9. Count the radioactivity for 1 min using a gamma counter (Cobra II auto-gamma counter, Packward Instrument Co., Model D 5005).

Appendix D. Procedure for enzyme-linked immunsorbent assay (ELISA)

A. Preparation of reagents

1. 10x Phosphate buffered saline (PBS) solution

   80 g NaCl, 12.07 g Na2HPO4, 20 g KH2PO4,

   2.0 g KCl, 2.0g sodium azide

   - Bring to near 1,000 ml with deionized water.
   - Adjust pH to 6.8.
   - Bring to 1,000 ml with deionized water.
   - Store at 4°C

2. 10% Bovine serum albumin (BSA) solution

   25 g bovine serum albumin

   25 ml 10x PBS

   - Bring to 250 ml with deionzed water.
   - Stir until dissolved at 4°C.
   - Sterilize through 0.2 nm filter
   - Store at 4°C

3. 10x Tris-buffered saline (TBS) solution

350.64 g NaCl, 96.88 g Tris, 8.0 g sodium azide

- Bring to near 4,000 ml with deionized water.
- Adjust pH to 7.4.
- Bring to 4,000 ml with deionized water.
- Store at room temperature.

4. Glassware treatment solution

10 ml 10% BSA, 90 ml 10x PBS

- Bring to 1,000 ml with deionized water.
- Store at 4°C.

5. Washing buffer

400 ml 10x TBS, 20 ml 10% Tween 20

- Bring to 4,000 ml with deionized water.
- Store at room temperature.

6. 1x Diluent solution

100 ml 10% BSA, 90 ml 10x PBS,

5 ml 10% Tween 20, 5 mg Bromophenol blue

- Bring to 1,000 ml with deionized water.
- Store at 4°C.

7. Tris coating buffer solution

2.44 g Tris, 5.84 g NaCl, 0.2 g sodium azide

- Bring to 1,000 ml with deionized water.
- Adjust pH to 8.5.
- Store at 4°C.

8. Standard solution

- For standards, porcine CBG from pig serum pool was isolated and purified by affinity chromatography and HPLC-DEAE anion exchange technique and adjusted to 12.15 ng/ul by dilution with 1x PBS.
- Standard stock solution was perpared in bulk and aliquoted with 1x PBS.
- Standard concentrations are:

ng/well	ng/ul	pCBG stock (uL)	1x PBS (uL)
400	8.0	698.2	301.8
160	3.2	263.3	736.7
50	1.0	82.3	917.7
20	0.4	32.9	967.1
8	0.16	13.2	986.8
2.5	0.05	4.1	995.9
1.0	0.02	1.7	998.3
0.3	0.006	0.5	999.5

9. CBG antibody stock solution

   - Rabbit anti-pCBG was from a rabbit injected with CBG purified from porcine plasma.
   - A 1/1,000 dilution of anitiserum was prepared with 1x Diluent solution.
   - Store in 250 ul aliquots in screw cap vial and frozen at -70°C.

10. CBG coating solution for 50 plates

    8,227.74 ul pCBG stock solution (12.154 ng/ul)

    491,772.26 ul Tris coating buffer

   - Prepare immediately before use.

11. Substrate solution

   - For every 5 mls of substrate, mix 1 ml of diethanolamine buffer (5x), 4 ml of deionized water, and 1 tablet of p-nitrophenyl-phosphate.
   - Agitate gently to dissolve the tablet.
   - Prepare the buffer just before use.

B. Procedure

1. Coat microtiter plate (Corning 96 well ELISA plate, NY)with purified porcine CBG (20 ng/well) by incubating at 4°C for 48 hr.
2. Concurrently, in a separate plate previously blocked with glassware treatment solution, incubate samples (2.5 ul/well), reference porcine (2.5 ul/well), 1x diluent control (2.5 ul/well) or standard porcine CBG in 1x PBS solution with rabbit anti-pCBG serum at final dilution of 1/200,000 at 4°C for 48 hr.
3. Wash the pCBG coated plate with washing buffer on the plate washer (Microplate Autowashe, Bio-Tek Instruments, Inc., Winooski, VT) and tap dry on paper towels.
4. Transfer the sample-antibody mixture (100 ul/well) to the corresponding wells of the coated plate and incubate in a humidified chamber at 30°C for 2 hr.
5. Wash the plate on the plate washer and tap dry.
6. Add 100 ul/well of anti-rabbit IgG alkaline phosphatase conjugate in a 1/20,000 dilution with 1x Diluent to the washed plate and incubate in a humidified chamber at 30°C for 2 hr.
7. Wash the plate on the plate washer and tap dry.
8. Add 100 ul/well of the phosphate substrate to the washed plate and incubate in a humidified chamber at 30°C for 1 hr.
9. Stop the reaction with an equal volume of 5% Na_2EDTA solution.

10. Read the resultant color development at 405 nm on a Bio Tek automated Micro plate Reader (Bio Tek Instruments, Inc., Winooski, VT).
11. Calculate the amount of CBG in the sample using a four parameter fit, kineticalic softwafer (Bio Tek Instruments, Inc., Winooski, VT)

Appendix E. Procedure for slot blot analysis

A. Preparation of Reagents

1. 20x SSPE treated with 0.1% DEPC

   - Dissolve 175.3 g of NaCl, 27.6 g of NaH2PO4.H2O and 7.4 g of EDTA in 800 ml of distilled water.
   - Adjust the pH to 7.4 with 10 N NaOH.
   - Adjust the volume to 1 liter with distilled water.
   - Add 1 ml of DEPC solution.
   - Stir well and stand overnight at room temperature.
   - Sterilize by autoclaving and store at room temperature

2. 50x Denhardt's solution

   5 g Ficoll (Type 400, Pharmacia)

   5 g polyvinylpyrrolidone

   5 g bovine serum albumin (Pentex Fraction V)

   - Dissolve deionized water to 500 ml
   - Filter and stored at -20°C

3. Prehybridization solution

5x SSPE-DEPC	5 ml of 20x SSPE-DEPC soltuion
50% formamide	10 ml of deionized formamide

water-DEPC	4 ml
2x Denhardt's	0.8 ml of 50x Denhardt's solution
0.2% SDS	0.2 ml of 20% SDS solution
Yeast total RNA(300 ug/ml)	6 mg

- Stir the mixture to dissolve yeast total RNA well.

B. Procedure

1. Prepare the loading sample as described below:

20 ug of total RNA sample	X ul
deionized water treated with 0.1% DEPC	(100 - X) ul
Deionized formaldehyde	150 ul
20x SSPE treated with 0.1% DEPC	150 ul
total final volume of the loading sample	400 ul

- Incubate the mixture at 65°C for 15 min
- Chill the mixture on ice for 5 min.

2. Assemble the MINIFOLD II cassette (Scheicher & Schuell, Keene, NH) as described below.
   - Float Nytran super charge membrane (Scheicher & Schuell, Keene, NH) on 100 ml of water treated with 0.1% DEPC for 10 min

- Soak the membrane in 200 ml of 10x SSPE treated with 0.1% DEPC for 10 min.
- Soak two piece of Whattman paper in 200 ml of 10x SSPE -DEPC for a few sec.
- Assemble the cassete.

3. Load 500 ul of 10x SSPE-DEPC to all wells and vaccum out.
4. Load 400 ul of samples and vaccum out.
5. Load 550 ul of 10x SSPE-DEPC to all wells and vaccum out.
6. Cross-link the blot in UV crosslinker (1,200 kJ/m^2, Hoefer scientific instruments, San Francisco, CA) and store it at -20°C.
7. Incubate the blot in prehybridization solution at 42°C overnight.
8. Label porcine CBG cDNA probe with 60 uCi of [α-^{32}P] dCTP using a random primer labeling kit (Stratagene, La Jolla, CA).
9. Remove unreacted nucleotide through column containing 5% Sephadex G-50.
10. Count the radioactivity of the ^{32}P-labeled CBG cDNA probe and calculate the volume for 4 x 10^7 cpm.
11. Denature the ^{32}P-labeled CBG cDNA probe by heating at 95°C for 10 min and then add it to the prehybridization solution (2 x 10^6 cpm/ml).
12. Incubate the blot in the hybridization solution at 42°C overnight.
13. Wash the blot twice with 500 ml of 2 x SSPE containing 0.1% SDS at room temperature for 5 min.

14. Wash the blot once with 500 ml of 1 x SSPE containing 0.1% SDS at room temperature for 10 min
15. Wash the blot once with 500 ml of 1 x SSPE containing 0.1% SDS at 65°C for 10 min.
16. Wash the blot once with 500 ml of 0.1 x SSPE containing 0.1 % SDS at 65°C for 20 min.
17. Expose the blot to Kodak X-Omat film at -70°C for 24 hr to detect the radioactivity exposed from the radiolabeled porcine CBG probe.
18. For reprobing the blot with human β-actin cDNA, strip off the porcine CBG cDNA probe by the boiling the blot in 600 ml of 0.2 % SDS treated with 0.1% DEPC for 20 min, and then repeat step 15 through 25.
19. Radioactivity is measured using an UltraScan XL Laser Densitometer (LKB Produkter AB, Bromma, Sweden).
20. The expression level of porcine CBG mRNA is expressed relative to β-actin mRNA level.

VITA

Jeonghoon Heo was born in Cheju city, Korea on August 19, 1964. In March of 1983 he entered the Kyung-Hee University and in February of 1987 received the degree of Bachelor of Science in Biology. The following March he entered the Kyung-Hee University and in February of 1989 received a Master of Science degree in Zoology. In August of 1994 he enrolled in graduate school at the University of Tennessee, Knoxville in the Department of Animal Science and in December of 1996 received a Master of Science degree in Animal Science. The following January he entered the Doctor of Philosophy program at the University of Tennessee, Knoxville and in August of 2001 received his Doctor of Philosophy Degree in Animal Science under the guidance of Dr. Henry G. Kattesh, Ph. D.

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